DECLARATION OF DR. DONALD L. KREUTZER UNDER 37 C.F.R. 1.132

I, Donald L. Kreutzer, declare:

1. I am a Professor of Surgery and Director of the Center for Molecular Tissue Engineering, at the University of Connecticut School of Medicine in Farmington, Connecticut. I have 35 years of experience in the fields of tissue engineering, tissue injury, inflammation, immunopathology and diabetes research. During the past 10 years, the primary focus of my research has been on the development of commercially viable glucose sensors.

2. Diabetes is a chronic disease that afflicts over 20 million people in the United States alone, with an annual cost in the U.S. of over $150 billion in direct and indirect expenditures [1]. The public health significance of diabetes is manifested in the various long-term complications resulting in premature death, disability and a compromised quality of life [2]. Central to minimizing these devastating complications, and their associate health and economic costs, is maintaining normal blood glucose levels. Currently, repeated "finger sticks" to obtain capillary blood samples is the major approach to monitoring blood glucose levels. Because of the pain and inconvenience of this procedure, patient compliance is often poor. Thus, there is a significant need for a method that would allow long-term continuous blood glucose monitoring. Implantable glucose sensors hold significant promise to fill this critical clinical need for people with diabetes.

3. ***Unfortunately, although the concept of implantable sensors has existed for over 30 years; there has only been limited success in developing a viable implantable glucose sensor that lasts <u>consistently</u> for more than a few days*** [3-8]. ***Although, there are short duration transdermal glucose sensors on the market, they are only approved for up to 7 days. Generally, both health insurers and governmental agencies have not been willing to pay for these short duration implantable glucose sensors.*** Clearly, developing sensors with extended life spans would be more appealing to the health care community. Additionally, glucose sensors with extended lifespan hold the promise of "closed-loop" systems that would utilize these sensors with insulin pumps (artificial pancreases). The importance of tissue responses to sensor function is underscored by a recent NIH report that states: "***The major barrier in sensor development and deployment is the loss of sensor function when put in contact with a biological system such as blood, saliva or interstitial fluid. This is referred to as biofouling. It is critical to overcome this natural protective barrier with research designed at understanding the interaction of proteins and cells with the sensor surface (immune reactions) and the subsequent process of fibrotic encapsulation of the sensor.***" [9]. We have found that gels and matrices with and without integrated tissue response modifiers extend

glucose sensor function *in vivo*. This extension of sensor lifespan *in vivo* will dramatically enhance quality of life and reduce complication for patient with diabetes.

4. Based on our previous and current data, we expect that the first commercial application of our coating technology will be in the form of a basement membrane coated implantable glucose sensor. We have demonstrated that coating the portion of implantable glucose sensors that enters the body dramatically extends the functional lifespan of the sensor from 5 days to over 2 weeks in our mouse model. We believe this exciting data is consistent with the disclosure and claims of our patent application. We expect that the coated sensor will be useful for patients with diabetes. The coated sensors also will be useful for diabetic pets (e.g. dogs and cats).

5. In addition to glucose sensors coated with basement membrane alone, our patent application describes sensors coated with basement membrane supplemented with growth factors that enhance the growth of vascular networks (e.g. blood vessels). These vascular networks are critical to maximizing the rapid movement of blood (i.e. glucose) through the tissue and thereby giving a more real time blood glucose values. Initially it is likely that the growth factors for the vascular endothelial cell growth factor (VEGF) family will be used (i.e. VEGF-A, VEGF-B, VEGF-C, and VEGF-D) individually or in combination. This first generation of matrix enhanced glucose sensor will further extend glucose sensor function. Further embodiments involve the use of adult stem cells added to growth factor supplemented basement membrane to further extend sensor lifespan by providing long-term production of stem cell derived VEGF related growth factors which would sustain vascular networks at sites of glucose sensor implantation for months to potential years.

6. In 2005, we published an article in the leading international diabetes research and technology journal, *Diabetes Technology & Therapeutics (D T & T)*. Our article appears in *D T & T*, vol., 7, no, 5, pp. 727-737, and describes a murine model for implantable glucose sensors and use of the model in developing longer-lasting glucose sensors for human patients. A copy of the article is attached as **Exhibit 1**. A commentary about our research findings was prepared by Colonel Karl E. Friedl, Ph.D., U.S. Army Research Institute of Environmental Medicine, Natick, MA, was also published in the same edition of *D T & T*, and is attached as **Exhibit 2**. In his analysis, Dr. Friedl indicates that due to the prevalence of diabetes in the US, implantable glucose sensors are a current subject of the Army's Technologies of Metabolic Monitoring research program. Dr. Friedl states that "[b]iocompatibility is now the principal impediment to long-term glucose sensing. The remarkable defenses that thwart sustained sensor performance range from direct cellular attacks on sensor membranes that render the systems inoperable within hours or days, to fibrotic encapsulation and isolation of the sensors that prevent real-time

measurement of blood glucose changes." Analysis, *Diabetes Technology & Therapeutics*, Vol 7, No. 5 (2005) pp 738-740, 738. Dr. Friedl's comments substantiate my understanding as a researcher in the field of diabetes that there is an important medical need for glucose sensors having improved function and lifespan.

8. In 2006, we co-authored an article in *Diabetes Technology & Therapeutics* with researchers from Abbott Diabetes Care, Alameda, CA. The article, attached as **Exhibit 3**, describes a complete system for real-time continuous subcutaneous glucose monitoring. Continuous monitoring of blood sugar levels offers substantial health benefits to patients with diabetes. Conventional monitoring of blood glucose levels involves measuring blood glucose content four times daily. It is well recognized that the "key factor in preventing the devastating complication and death associated with diabetes in the close monitoring of blood glucose levels. . . . Unfortunately, because of the pain and inconvenience associated with 'finger sticking,' patient compliance is often poor. Even with good patient compliance and regular blood glucose testing, blood glucose swings and 'excursions' are often undetected and directly contribute to the complications and deaths associated with diabetes. . . . Clearly, there is a critical need for a method that would allow long-term continuous blood glucose monitoring in vivo." *Diabetes Technology & Therapeutics,* vol. 8, no. 3 (2006) pp. 402-412, 403.

9. Dr. Friedl wrote a commentary on the second paper that also appeared in the 2006 journal. The article is attached as **Exhibit 4**. Dr. Friedl characterizes the monitoring system described in our work as providing "an efficient approach to testing new therapies" and indicates that the implant system "will itself be a test model for improvement in implantable systems, including studies of the interactions of implants and specific genetic traits and physiological conditions." He further indicated that "[t]he most important indicator of success for [the model described in our article] will be that patients and soldiers realize the benefits of new discoveries in metabolic research sooner." *Diabetes Technology & Therapeutics,* vol. 8, no. 3 (2006) pp. 413-414, 414. Thus, it is clear that there is substantial interest in the medical community for diabetes monitoring and implantation research, including our work in this area.

10. In the prosecution of U.S. Patent Application 10/578,171, the claims stand rejected on the basis of U.S. Patent No. 6,673,596, Sayler, alone or in combination with U.S. Patent No. 6,824,561 to Soykan. I have reviewed both of the cited patents.

11. The Sayler Patent describes a bioluminescent bioreporter integrated circuit device (BBIC) configured to detect analytes in fluid when implanted in the body of an animal. The BBIC contains a biological component (whole cells) that functions as a bioreporter, and an analytical

measuring element, such as an integrated circuit. The bioreporter emits light when it is exposed to an analyte. The Sayler patent uses the word "container" when referring to the "biocompatible container" and also when referring the "bioreporter container." The two containers are different from one another. The "biocompatible container" is the container that contains the entire implantable device (col. 3, lines 39-42), and is designated as 20 on Fig. 2. The "bioreporter container" is the container for the bio-reporter and is designated as 22 on Fig. 2. Furthermore, the Sayler patent uses the term "biocompatible housing" (col, 6, line 11 and Fig. 2) interchangeably with biocompatible container (col. 3, lines 40-43). For clarification purposes, I refer to the biocompatible container as the "biocompatible container/housing."

12. In Sayler, the biocompatible container/housing 20 is described in cols. 3, 6, 10 (lines 46-48), 17 and 25, and the bioreporter container 22 is described in cols. 4, 10 (lines 42-46), 23, 31 and 35. The bioreporter container also is described in claims 2-4 of Sayler. Each of these portions of Sayler is discussed below. The relevant portions of Sayler are considered in the order in which they appear in the text of Sayler, except for Sayler's disclosure of Matrigel, which is discussed first below. Matrigel is a cell culture derived basement membrane material. Matrigel is used in the Examples of our application, and cell culture derived basement membrane is claimed in independent claims 37 and 78 of our application.

13. The only specific disclosure of any type of basement membrane in Sayler is in **Example 7, col. 35**. This portion of Sayler indicates that cell culture derived basement membrane (Matrigel) is used **inside** the bioreporter device, **not as a surface coating in contact with body tissue** (see col. 35, lines 42-48). More particularly, Matrigel is employed as a glue to support cells in compartment 22 to promote the sensing function of the device. Col. 35, lines 42-48 of Sayler state: "*Biochips may be coated with Matrigel, a basement membrane material that promotes attachment of epithelial cells. An alternative approach suspends the cells in Matrigel and allows it to form a gel on the surface of the biochip. The cells are then immobilized in the basement membrane material and are not subject to dislodgement by friction.*" In Sayler, the Matrigel clearly is not in contact with the biological tissue in which the BBIC is implanted. The reference to attachment of Matrigel to epithelial cells is not suggesting that the Matrigel would attach to body tissue in the Sayler embodiment, but instead explains why the Matrigel will function as a glue to hold the irradiated human tumor cells, hepG2 and Hela, in place on the biochip. The hepG2 and Hela cells are tumorgenic (i.e. the cause tumors when injected into tissue) which is why they are irradiated to prevent them from dividing, which is what tumor cells want to do. Additionally, irradiation, at levels that allow cell survival, cannot guarantee that 100% will not divide, thus it is possible that dividing tumor cells could survive the irradiation dose used for this type of procedure. A semi-permeable membrane is a membrane that allows passage of certain

molecules or ions, especially small molecules or ions, but acts as a barrier to larger substances. One would need to use a semi-permeable membrane to cover the cells in the embodiment described in col. 35 of Sayler for a number of reasons:

> First, the cells are **human tumors** and you would not want them getting out of the device and causing cancer in the body tissue. In fact it is known that cells, including tumors, can migrate through basement membranes *in vivo* and through Matrigel *in vitro* (BD BioScience) and thus without a semi-permeable membrane between the body tissue and the cells (attached by Matrigel), the reporter cells including tumor cells can migrate away from the reporter chip, resulting in loss of bio-reporter function. In the absence of a semi-permeable membrane the tumorgenic tumor cells can invade the host tissue and potentially result in tumor formation.
>
> Second, without a semi-permeable membrane on the bioreporter chip, the tumor cells will cause blood vessels to grow into the reporter chip and interfere with reporter function, i.e. the red blood cells will interfere with the fluorescence measurements, and the blood vessels will mechanically dislodge the tumor cell for the reporter surface. (In contrast, as recited in the independent claims in our application, the growth of vessels **enhances** the function of our sensor by bringing blood close to the sensor.)
>
> Third, the semi-permeable membrane would be needed to prevent at least some of the cells (whether or not they are tumor cells) from being killed by the body's immune system (by innate and acquired immunity).
>
> Fourth,, if the chip was placed on the surface of a endoscope (as Sayler proposes as one possible use) and was used to hunt for tumor cells inside a human body, one would need a semi-permeable membrane so that the cells would not be rubbed off the device and deposited in the body. If deposition occurred the device would not function and again one would run the risk of inducing cancer at the sites where the tumor cells were "rubbed off" the endoscopic device.

Matrigel does not function as a semi-permeable membrane and therefore could not be used as the semi-permeable membrane in the embodiment described in col. 35 of Sayler. Thus, col. 35 of Sayler does not disclose, and furthermore **teaches away from**, the use of Matrigel to

encapsulate the bioreporter (cells) when no semi-permeable membrane is used, i.e. when the biological matrix (basement membrane) is in contact with **both** an outer surface of an implantable device **and also** with a biological system, as is recited in independent claims 1, 37 and 78 of our application. Furthermore, col. 35 of Sayler teaches away from the use of cells in a matrix to extend the functional lifespan of a sensor, as is recited in claims 1 and 28, and in the alternative in claim 37.

14. **Exhibit 5**, attached hereto, shows additional reasons why the Matrigel cell adhesive of Sayler cannot be in contact with both body tissue and the implant even if the cells in the bioreporter are not tumor cells. First, the sensor would not function due to interference by autofluoroescence (akin to the difficulty of seeing a polar bear (fluorescence of bioreporter) in a snowstorm (tissue autofluoroescence)). Matrigel itself has autofluoroescence, and entrapping the cells in Matrigel is akin to throwing a white tent over the polar bear in a snow storm. Furthermore, as indicated above, Matrigel is not a semi-permeable membrane but rather a gel or paste. If an attempt were made to use Matrigel as a semi-permeable membrane in a bioreporter system involving fluorescence cell reporters, the result would be failure of the bioreporter and device because of the following effects: **1)** tissue injury induced by the implantation of the device would cause inflammation which in turn would degrade the Matrigel and result in destruction of the bio-reporter cells as a result of invading inflammatory cells; **2)** Matrigel would not prevent leakage of toxic materials out of the sensor into the tissue, i.e., the toxic substances would kill or injure tissue cells, resulting in even more inflammation and fibrosis resulting in destruction of the bioreporter; **3)** Matrigel would not prevent the migration of the fluorescence cells themselves out of the bioreporter, **4)** Matrigel would not prevent the invasion of blood vessels into the bioreporter resulting in both the dislodging of the fluorescent bioreporter cells from the surface, as well as introducing blood into the bioreporter chamber which would block detection of the fluorescence bioreporter cells; **5)** Matrigel would not block the movement of antibodies or complement proteins into the bioreporter, resulting in destruction of the reporter cells directly and promoting complement and leukocyte mediated cell reporter cell killing; **6)** Matrigel would not block complement, antibody or leukocyte bystander killing of nearby tissue cells, resulting in release of toxic substance form the reporter cells; **7)** pus would accumulate in the sensor. Pus contains leukocytes, which would kill both reporter cells and nearby tissue cells, again triggering more inflammation and the release of toxic substance from the reporter cells; and **8)** the leukocytes also contain/release powerful proteases which would rapidly destroy the matrigel and the bio-reporter cells.

15. According to **col. 3, lines 39-42** of Sayler, the implantable device "[p]referably is contained in a biocompatible container[/housing]." Col. 3, lines 43-44 state that the biocompatible

container/housing can comprise (a) silicon nitride, (b) silicon oxide, or (c) a suitable **polymer matrix,** such as polyvinyl alcohol, poly-L-lysine or alginate. According to claim 2 of Sayler, if a polymeric matrix is used, the function of the matrix is to keep the bioreporter cells in place over the integrated circuit. According to col. 3, lines 46-48, the polymer matrix may also further comprise a microporous hydrogel, a mesh-reinforced hydrogel or a filter-supported hydrogel. Basement membrane is NOT mentioned in this part of Sayler and thus this portion of Sayler does not anticipate or render obvious independent claims 37 and 78, both of which recite cell culture derived basement membrane. As explained above, Matrigel does not function as a semi-permeable membrane as this term is used in Sayler. Furthermore, this portion of Sayler does not disclose the presence of **cells** in a matrix, and therefore does not anticipate or render obvious independent claims 1, 28 and 37 of our application, all of which recite the presence of cells in the matrix.

16. The bioreporter container is described in cols. 4 and 6 of Sayler. More particularly, Sayler provides that in order for the BBIC to function, either the bioreporter container must include a semi-permeable membrane **(col. 4, lines 52-59)**, or the portion of the biocompatible container/housing that covers the bioreporter container must include a semi-permeable membrane **(col. 6, lines 11-18)**. The semi-permeable membrane is constructed to allow the analyte to enter the BBIC ***and*** to prevent the bioreporter cells and/or their toxic byproducts from exiting the BBIC. (Col. 4, lines 55-59 and col. 6, lines 13-18). Thus, in the embodiments of Sayler that are described in cols. 4 and 6, after implantation, the contents of the bioreporter container are **separated from** body tissue by the semi-permeable membrane and in some cases also (or instead) by the biocompatible container/housing. Stated another way, in the first case, the contents of the bioreporter container are separated from body tissue by a semi-permeable membrane that is part of the bioreporter container. In the second case, the contents of the bioreporter container are separated from body tissue by a semi-permeable membrane that is part of the biocompatible container/housing. In the third case, there is a first semi-permeable membrane that is part of the biocompatible container/housing and a second semi-permeable membrane that is part of the bioreporter container.

17. **Col. 6, lines 18-24** of Sayler provide that "[t]he *bioreporter may be in solution, that is a cell suspension, and entrapped in the container by the semi-permeable membrane, or alternatively the bioreporter may be encapsulated in a selectively permeable polymer matrix that is capable of allowing the selected substance in solution reach the bioreporter. Preferably, the matrix is optically clear."* This portion of Sayler indicates that if there is a semi-permeable membrane holding the bioreporter cells in the bioreporter container, a selectively permeable matrix is not needed. However, if there is no semi-permeable membrane holding the bioreporter cells in the

bioreporter container, a selectively permeable polymer matrix is required "that is capable of allowing the selected substance in solution to reach the bioreporter." This embodiment is also described in claims 2-4 of Sayler. Fig. 2 of Sayler depicts a semi-permeable membrane 21 (col. 10, line 65) which prevents bioreporter cells from leaving the bioreporter container (col. 4, lines 55-59) and Fig. 10C of Sayler shows an optional "selectively permeable membrane." While Sayler does not define "selectively permeable matrix," this term generally refers to a matrix that will allow only specific types of molecules through. Thus, in Sayler, either a semi-membrane layer, or a selectively permeable matrix that performs essentially the same function as a semi-permeable membrane, is used to keep out toxins, antibodies, pus, etc., and is positioned between body tissue and the bioreporter cells. In contrast, independent claims 1, 37 and 78 of the present application provide that a cell-containing biological matrix is **in contact with** both the implantable device and biological system and therefore there is no semi-permeable membrane or selectively permeable matrix separating the implantable device from the biological system. Claims 37 and 78 provide that the biological matrix comprises cell culture derived basement membrane, which is not a semi-permeable membrane or a selectively permeable matrix, because it will let all sizes of molecules through. Claims 1 and 28 of our application (and claim 37 in the alternative) provide that the cells in the matrix extend the functional lifespan of the sensor, which is not the case in Sayler.

18. **Col. 10, lines 42-48** of Sayler provide that the bioreporters are "*entrapped in a container behind a semi-permeable membrane*" or are "*encased in a polymer matrix.*" This portion of Sayler then further states that *"[t]he BBIC is enclosed in a biocompatible housing with a semi-permeable membrane covering the bioreporter region. This membrane allows glucose to pass to the bioreporters, yet stops the passage of larger molecules that could interfere with the glucose measurement.*" (emphasis added). Col. 10 of Sayler does not disclose or suggest an implant system in which a biological matrix is in contact with both an implantable device and biological (body) tissue (our claims 1, 37 and 78). Furthermore, this portion of Sayler does not describe a matrix (in contact with an outer surface of an implantable device) containing cells that extend the functional lifespan of a sensor (our claim 28).

19. **Column 17, lines 46-67** of Sayler discuss biocompatible coverings and coatings for implants and prosthetic devices that can be used to either coat or form the housing. Suitable materials that are mentioned to minimize capsule formation and prevent physiological rejection of the implant include a thin biocompatible carbon film, a three-dimensionally woven or knitted fabric of organic fibers, the coverings disclosed in US. Patent Nos. 5,653,755, 5,779,734 and 5,814,091. Collagen coating, and albumin coating. U.S. Patent Nos. 5,653,755 and 5,779,734 describe implant coverings made from fluoropolymer filaments attached to a stretch fabric backing. U.S. Patent

No. 5,814,091 describes a two layer capsule for a medical implant comprising a first layer of a biocompatible material such as titanium and a second layer of a substantially diffusion-proof and corrosion-resistant metal. None of the materials described in this part of Sayler constitutes a "biological matrix" supporting "a plurality of cells", as is recited in independent claims 1, 28 and 37. Furthermore, none of the materials described in this portion of Sayler constitutes a plurality of cells associated with a cell culture derived basement membrane, as is recited in independent claims 37. Finally, none of the listed materials in this portion of Sayler constitutes "cell culture derived basement membrane", as is recited in independent claim 78. [10]. A useful analogy to distinguish between naturally occurring basement membrane and cell culture derived basement membrane is the following: if naturally occurring basement membrane is considered to have an order 3D structure with specific composition and layers i.e. a "orderly brick wall with specific 3D structure," cell culture derived basement membrane that is **extracted** from cultured cells in vitro would be viewed as "a loose pile of bricks", i.e. the pile of bricks has some of the same components as the wall, but the bricks have lost their specific (3D) relationship to each other, analogous to some of their function. Thus, column 17 of Sayler clearly does not disclose or suggest the cell culture derived basement membrane recited in independent claims 37 and 78 of the present application.

20. As indicated above, Matrigel is a cell culture derived basement membrane. Sayler does not disclose or suggest the use of Matrigel to form, cover or coat the biocompatible container/housing. The only use of Matrigel in Sayler is ***inside*** the bioreporter container, as is explained above in paragraph 13.

21. **Col. 23, lines 32-56 of Sayler** refer to two different embodiments. One embodiment is a biosensor consisting of bioengineered cells entrapped in suspension behind a semi-permeable membrane. This first embodiment is shown in Fig. 10C (Fig. 10C has a notation of an optional membrane). The second embodiment is a biosensor consisting of cells encapsulated in a matrix. The second embodiment either is shown in Fig. 10B, is the version of Fig. 10C that does not include the membrane, or is not depicted in the drawings. Independent claims 37 and 78 of our patent application provide that the membrane which contacts the outer surface of the implant **is cell culture derived basement membrane.** Cell culture derived basement membrane is NOT a "semi-permeable membrane" as this term is used in connection with the first embodiment of Sayler, because it would not keep fluorescent materials inside the sensor of Sayler and would not keep antibodies and pus out of the sensor of Sayler. Moreover, the cell culture derived Matrigel used to support cells **inside** the semi-permeable membrane 21 of Sayler (as described at col. 35, lines 39-48 of Sayler) is not "in contact with an outer surface of the implantable device" as is required in our claims 1, 37 and 78 because the outer surface of this portion of the BBIC requires

a membrane or matrix to keep out antibodies and other bio-reporter toxic substances, and does not contain cells that extend the functional lifespan of the sensor (our claim 28).

22. The second embodiment of Sayler described at **column 23, lines 32-56** provides that as an alternative to the use of a semi-permeable membrane, the biosensor may "consist of cells encapsulated in a polymeric matrix." Suitable matrices that are listed in Sayler are polyvinyl alcohol, sol-gel and alginate. Lines 52-55 in col. 23 of Sayler specifically mention polyvinyl alcohol mesh reinforced or microporous filter supported hydrogels. This portion of Sayler acknowledges that when no semi-permeable membrane is used, the encapsulating material must function as a membrane that protects the bioreporter from substances in the tissue and protects the tissue from toxic substances originating in the bioreporter. As indicated above, cell culture derived basement membrane would not function as a semi-permeable membrane as the term "semi-permeable membrane" is defined in Sayler, and thus Sayler does not disclose, and teaches away from, the use of cell culture derived basement membrane, as is recited in our claims 37 and 78. Furthermore, this portion of Sayler does not disclose or suggest a biological matrix in contact with both biological tissue and an implantable device, as is recited in our claims 1, 37 and 78. Finally, this portion of Sayler does not disclose or suggest a matrix containing cells that extend the functional lifespan of a sensor, as in recited in our claims 1 and 28, and in the alternative in claim 37.

23. **Col. 25, lines 14-26** of Sayler state that host rejection effects can be minimized by enclosing cells in hydrogel membranes. Sayler states that the hydrogels "block access by the humoral and cellular components of the host's immune system but will remain permeable to the target substance glucose. Matrigel and cell culture derived basement membrane cannot be encompassed in this description of hydrogel membranes because Matrigel will not block access by antibodies, and is not likely to block the access of immune cells for any length of time, but instead will simply slow the entry of immune cells if a thick layer is used. Matrigel is a jelly-like substance, and antibodies will go right thru this type of matrix. Examples of suitable hydrogel materials provided at col. 25, lines 22-25 of Sayler are mesh-reinforced polyvinyl alcohol hydrogel bags. Col. 25, lines 26-37 mention that it may be necessary to provide a barrier between the cells and the appropriate body fluid. There is no mention of basement membrane or cell culture derived basement membrane in col. 25 of Sayler. Furthermore, there is no mention in col. 25 of Sayler of a matrix containing cells that extend the functional lifespan of a sensor.

24. **Col. 31, lines 33-37** of Sayler state that "[i]t may be necessary to isolate the bioreporters using a semi-permeable membrane to allow the transport of small molecules such as glucose and insulin across the membrane and prohibit the influx of immune effector cells and antibodies. . . .

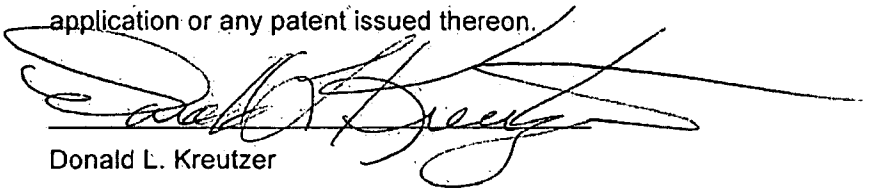
However, small molecules such as cytokines can still enter the selective membranes and interfere with the bioluminescent reporter cell lines. This approach has been used extensively by those of skill in the art." The quoted portion of Sayler further supports the teaching in Sayler that either a semi-permeable membrane, or a "selectively permeable membrane" (or "selectively permeable matrix") that functions as a semi-permeable membrane, is required to keep unwanted materials out of the bioreporter portion of the sensor.

25. As mentioned above, an important distinction of our system over each portion of Sayler directed to the biocompatible container/housing, is that the cells in the matrix of our system, as recited in claims 1 and 28, and as recited in the alternative in claim 37, **extend the functional lifespan of the sensor.** The biological materials described in Sayler (at col. 17, lines 62-67 of Sayler) as promoting some type of biocompatibility between the implant and the body (although there is no mention of extended functional lifespan) are collagen and albumin, neither of which constitutes "cells" in a biological matrix (our claims 1, 28 and 37), and neither of which constitutes cell culture derived basement membrane (our claim 78). The hydrogel mentioned at col. 25, lines 14-25 of Sayler blocks access of components of the host's immune system, which is different from the structure and function of the cell culture derived basement membrane in our patent application.

26. The Soykan Patent is cited in connection with the rejection of independent claims 28 and 37. Soykan describes an implantable system for drug delivery to treat a disease, not to promote compatibility of an implant with body tissue. The Office Action indicates that Soykan is cited for its disclosure of endothelial cells that line the walls of blood vessels and that secrete vasodilatory, thrombolytic or angiogenic factors, such as vascular endothelial growth factor (VEGF). This document does not make up for the above-noted deficiencies of Sayler in that it does not disclose a biological matrix containing cells that extend the functional lifespan of a sensor, as is recited in claims 1 and 28 of our application. Instead, the cells extend the lifespan of the person in whom the device is implanted by treating coronary artery disease, cerebral vascular occlusion, or the like. Furthermore, Soykan does not disclose a matrix material comprising cell culture derived basement membrane that is in direct contact with both a sensor and body tissue as is recited in claims 37 and 78 of the present application.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Donald L. Kreutzer

March 9 , 2010
Date

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[10] General references to "collagen" in the literature are directed to fibrillar (Type I) collagen. In contrast, cell culture derived basement membrane contains various components, including laminin and nonfibrillar (usually Type IV) collagen.

Exhibit 1

DIABETES TECHNOLOGY & THERAPEUTICS
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Military Metabolic Monitoring
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Murine Model of Implantable Glucose Sensors: A Novel Model for Glucose Sensor Development

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ABSTRACT

Although implantable glucose sensors have existed for over 30 years, their function deteriorates in hours to days, in large part as a result of tissue responses to the implanted sensor (i.e., acute and chronic inflammation, fibrosis, and vessel regression). Little is known about the mediators and mechanisms that control these tissue responses to implantable glucose sensors. In the present study, we developed and validated a murine model for implantable glucose sensors, which suitably parallel sensor function in humans. Using special care in implantation and implant retaining techniques, we demonstrated that (1) sensor function deteriorates rapidly within days post-implantation and (2) loss of glucose sensor function correlated with tissue reactions at the sites of sensor implantation, especially in the vicinity of the glucose oxidase-based working electrode. These studies establish a murine model that can be used to evaluate implantable glucose sensors in vivo. This model should provide the foundation for future studies to understand the factors and mechanisms that control sensor function in vivo.

INTRODUCTION

THE SINGLE MOST IMPORTANT FACTOR in preventing complications of diabetes is the close monitoring of blood glucose levels. Implantable glucose sensors hold significant promise to fill this critical clinical need for patients with diabetes. The concept of an implantable sensor has existed for over 30 years, but there has only been limited success in developing a commercially viable implantable glucose sensor that lasts *consistently* for more than a few hours or days.[1–6] Even experimental glucose sensors have seen variable levels of success in functionality and lifespan in vivo.[7–9] While it is generally accepted that the loss of function is associated with sensor-induced tissue reactions, i.e., inflammation and fibrosis, little is known about the mediators and mechanisms that are responsible for this loss of glucose sensor function in vivo. This lack of information is in part due to (1) the limited number of in vivo studies, (2) lack of significant characterization of tissue reactions and correlation with sensor functions, and (3) the lack of a robust animal model that provides the

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foundation for a rational approach to enhance sensor design and in vivo function.

In this study we tested a standard amperometric glucose sensor in the mouse to characterize an in vivo model of implantable glucose sensors, which displays sensor function and tissue reactions seen in other mammals, including humans. This simple and effective murine model will likely provide the foundation for enhancing sensor function, by unraveling the mediators and mechanisms that control tissue reactions at sites of glucose sensor implantation.

MATERIALS AND METHODS

Glucose sensor fabrication and function in vitro

Fabrication and in vitro testing of our glucose oxidase-based amperometric glucose sensor (needle-type) were performed utilizing established protocols.[10–12] Briefly, the sensor consisted of a platinum (Pt) wire and a silver (Ag) wire, with the Teflon® (Dupont, Wilmington, DE) coating being removed from the wire's end. The working electrode consists of the bare Pt wire coiled 10 times around the insulated Pt wire. The Ag reference electrode wire was coiled approximately 15 times around the Pt wire proximal from the coiled Pt wire with a 2-mm gap between them. After the probe was sonicated in water for 5 min, silver chloride is formed on the Ag wire by applying a current of 0.04 mA in a stirred 0.1 *N* HCl solution for 60 min. Anodization and poly(*o*-phenylenediamine) film formation of the working electrode were performed as described previously.[13] Glucose oxidase (EC 1.1.3.4, from *Aspergillus niger*, 158 U/mg) was immobilized on the working electrode using glutaraldehyde (aqueous 25%) as a cross-linking agent and bovine serum albumin as a carrier protein. One microliter of that mixture was applied three times to the working electrode with a break of 30 min between dips to allow the solution to dry on the surface.[10] After the sensors were dip-coated with six layers of Nafion® (Aldrich, Milwaukee, WI), sensors were cured for 0.5 h at 120°C and stored dry at room temperature in closed containers.

In vitro calibrations of sensors were carried out at 37°C. The steady-state sensor current is measured with glucose concentrations of 0, 4.8, 13.9, 22.5, and 30.9 mmol/L in sterile phosphate-buffered saline solution. Sensors were stored in closed sterile containers until used for in vivo implantation and evaluation. All chemical reagents were obtained from Sigma (St. Louis, MO).

Glucose sensor implantation in the mouse

Animals utilized were ICR mice weighing 35–40 g from Harlan (Indianapolis, IN). These studies were approved by the Laboratory Animal Care Use Committee at the University of Connecticut, Farmington. Mice were shaved at least 24 h before sensor implantation. At the time of implantation, the shaved skin was disinfected using 70% isopropyl alcohol, and one sensor per mouse was implanted into the interscapular subcutaneous tissue. During the sensor implantation the mice were anesthesized with 1% isoflurane, and mouse body temperature was maintained at 37°C using a heating blanket. Prior to sensor implantation, 100–150 μL of injectable sterile, pyrogen-free, 0.9% NaCl was injected subcutaneously in the interscapular area of the mouse to provide an "implantation pocket." This implantation pocket was used to minimize tissue and sensor damage during sensor insertion. Next, a small opening was made in the "implantation pocket" using corneal scissors, and the sensor was then implanted in the subcutaneous "pocket" with the two sensor leads exposed (Fig. 1). The wound was closed with a drop of surgical glue (Veterinary Surgical Glue, Nexaband, Chicago, IL), and a small polyester mesh was placed on top of the exposed sensor leads (Fig. 1). The sensor leads and the nylon mesh were secured to the shaved mouse skin by applying a protective coating (New-Skin First Aid and Antiseptic Liquid Bandage, Medtech Corp., Jackson, WY). Mice were kept under anesthesia until the protective coating dried (aided by a low temperature blow dryer). The animals were housed individually to prevent dislodging of the sensor from aggressive behavior between the mice. Daily inspection of the sensor implantation site was necessary to prevent loss of mesh. All mice were maintained under specific pathogen-free conditions.

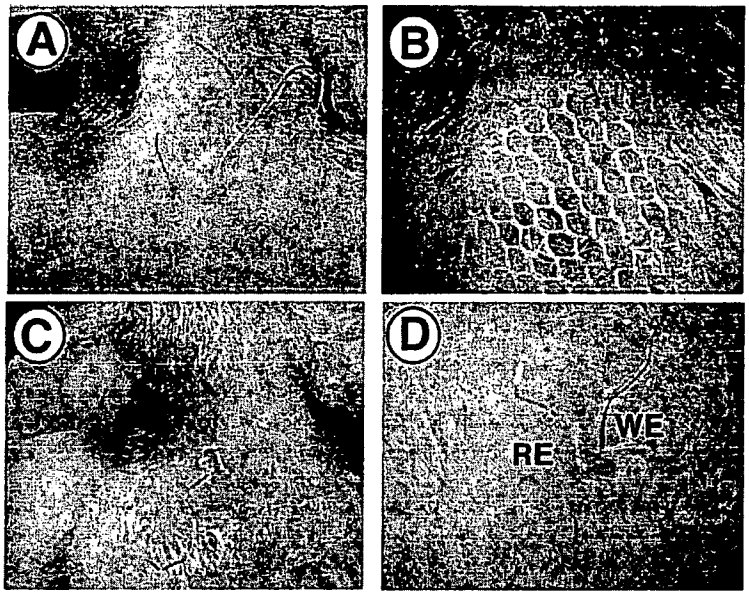

FIG. 1. Implantation of glucose sensors in ICR mice. Initially, a simple implantation procedure was developed that: (1) minimized implantation-associated trauma, (2) protected externalized sensor leads, and (3) minimized risk of infection. **A:** Sensor leads exposed 1 h post-sensor implantation. **B:** Sensor leads protected using nylon mesh. **C:** Exposed sensor leads 14 days post-sensor implantation. **D:** Close-up of exposed sensor leads at 14 days post-sensor implantation. RE, reference electrode; WE, working electrode.

Glucose sensor function in vivo

At predetermined time points after sensor implantation sensor function and blood glucose levels were assessed. For these studies animals were again anesthetized with 1% isofluorane, and maintained at 37°C using a heating blanket. The nylon mesh and protective coating were removed with acetone prior to sensor testing. To elevate blood glucose levels the animals were given 80–120 μL of a 0.5 g/mL dextrose solution intraperitoneally after a sensor baseline period of 20–30 min was established. To monitor blood glucose levels, a single drop of blood was obtained from the tail vein before glucose injection (baseline) and at 5, 10, and 20 min after glucose injection. Blood glucose levels were determined using a OneTouch® Ultra® glucose meter (Lifescan, Johnson & Johnson, Milpitas, CA). Implanted sensor performance was followed for about 40–60 min initially, and for at least 20–30 min at each subsequent testing session.

Glucose sensor function was tested in each mouse immediately following implantation, designated as 1 h post-implantation (HPI), 5 HPI, 1 day post-implantation (DPI), 2 DPI, 3 DPI, 7 DPI, 14 DPI, and occasionally up to 30 DPI. Between the sensor function tests, the mice were left unrestrained in their cages, without polarization of the sensor. A total of 24 mice were tested, each implanted with a different sensor. The two-point calibration method was used to calculate sensor sensitivity, expressed in nA/m*M*, as the ratio between the change in sensor response (in nA) and the change in blood glucose concentration (in m*M*) following an intraperitoneal glucose injection.[14]

Gross and histological evaluation

Sites of sensor implantation were evaluated grossly for redness, swelling, warmth, or other signs of inflammation both before and after removal of the protective nylon mesh. To evaluate the tissue responses to implantation of the glucose sensor at various time points, individual mice were euthanized, and tissue containing the implanted sensors was removed, fixed in 10% buffered formalin, and embedded in paraffin. The sensor was removed from tissue after paraffin embedding to minimize tissue damage from the removal, and then the tissue was re-embedded, processed for sectioning, and stained with hematoxylin and eosin or trichrome.[12] Histopathologic evaluation of tissue reactions at sites of sensor implantation was performed on mouse specimens obtained at 1 DPI, 3 DPI, 7 DPI, 14 DPI, and 1 month post-implantation of the glucose sensor. The tissue samples were examined for evidence of loss of cell and tissue architecture and signs of

acute and chronic inflammation including giant cell formation, necrosis, fibrosis, and vessel regression.

Quantification of tissue reactions at sensor implantation sites

Tissue surrounding the working electrode, the wire between the electrodes, and the reference electrode was evaluated histologically and given a Tissue Reaction Index or rank score between 0 and 4 for tissue reactions. Evaluation of tissue reactions included the presence of inflammation and or fibrosis at the implantation site. The tissue reaction index score for tissue reactions was analyzed as: 0 = no tissue reaction compared with normal (non-implanted sensor); 1 = slight and limited inflammation/fibrosis; 2 = mild to moderate inflammation/fibrosis; 3 = major inflammation/fibrosis; and 4 = severe and extensive inflammation/fibrosis.

Statistical analysis

For sensor response in vitro and in vivo the data are expressed in nA. All in vivo data obtained for these studies are expressed as mean ± SD values. Analysis of data for significance was done using Student's *t* test, with significance at $P \leq 0.01$.

RESULTS

Sensor implantation and gross appearance in the mouse model

One of the major hurtles in the successful development of the implanted glucose sensor model was to develop techniques to maintain placement and sterility of the implanted sensors in the mouse. To address these issues we implanted the sensor in the interscapular region of the neck, which prevented the individual mouse from directly removing the implanted sensor (Fig. 1). We found that injecting a small amount of saline subcutaneously at the site of sensor implantation created a simple pouch or "bleb" for sensor implantation that appears to markedly decrease tissue trauma and inflammation associated with the implantation procedure itself. We also used surgical glue to close the surgical wound at the implantation site, eliminating the need for sutures, which in itself causes major tissue reaction (Fig. 1A). Additionally, we found that utilizing nylon mesh to cover the exposed leads of the sensor and an antiseptic coating polymer (New Skin) both adhered the nylon mesh to the mouse skin and provided a sterile antiseptic coating to protect the surgical wound from infection (Fig. 1B). Using this technique there was no damage to the sensor leads by the mouse, and we had no wound infections. Figure 1C and D shows the protective mesh on the sensor and the exposed sensor leads 14 DPI.

Glucose sensor function in vivo

To validate our murine model we correlated blood glucose levels in the mouse with sensor function. Blood glucose levels paralleled sensor function at both 1 and 5 h post-sensor implantation (Fig. 2, 1h and 5h, respectively). Analysis of blood glucose and sensor function at 1 day post-sensor implantation clearly indicated that although the blood glucose levels were elevated after intraperitoneal injection of the glucose, there was little or no response from the implanted sensors (Fig. 2, 1d). Analysis of the blood glucose and sensor function at 2, 3, 7, 14, and 30 days post-sensor implantation consistently demonstrated that although blood glucose levels were elevated after intraperitoneal injection of glucose, there was no sensor function (Fig. 2, 2d, 3d, 7d, 14d, and 30d, respectively). This rapid loss of sensor sensitivity (Fig. 3) on 1 DPI was observed in virtually all sensors tested in the mouse model. Of the implanted glucose sensors we tested in the mouse model, most sensors lost most of their sensitivity within the first 24 h. However, one sensor implanted in vivo had still 50% of its initial sensor response at 14 DPI, and sensor sensitivity could be measured up to 18 DPI; this was extremely unusual behavior, and no other sensor implanted in any of the nearly 30 mice used in this study showed this kind of behavior. On that basis, we excluded this mouse in our summary graph (Fig. 3) since it did not reflect the general sensor pattern observed in the 24 mice included in this study. Other mice were

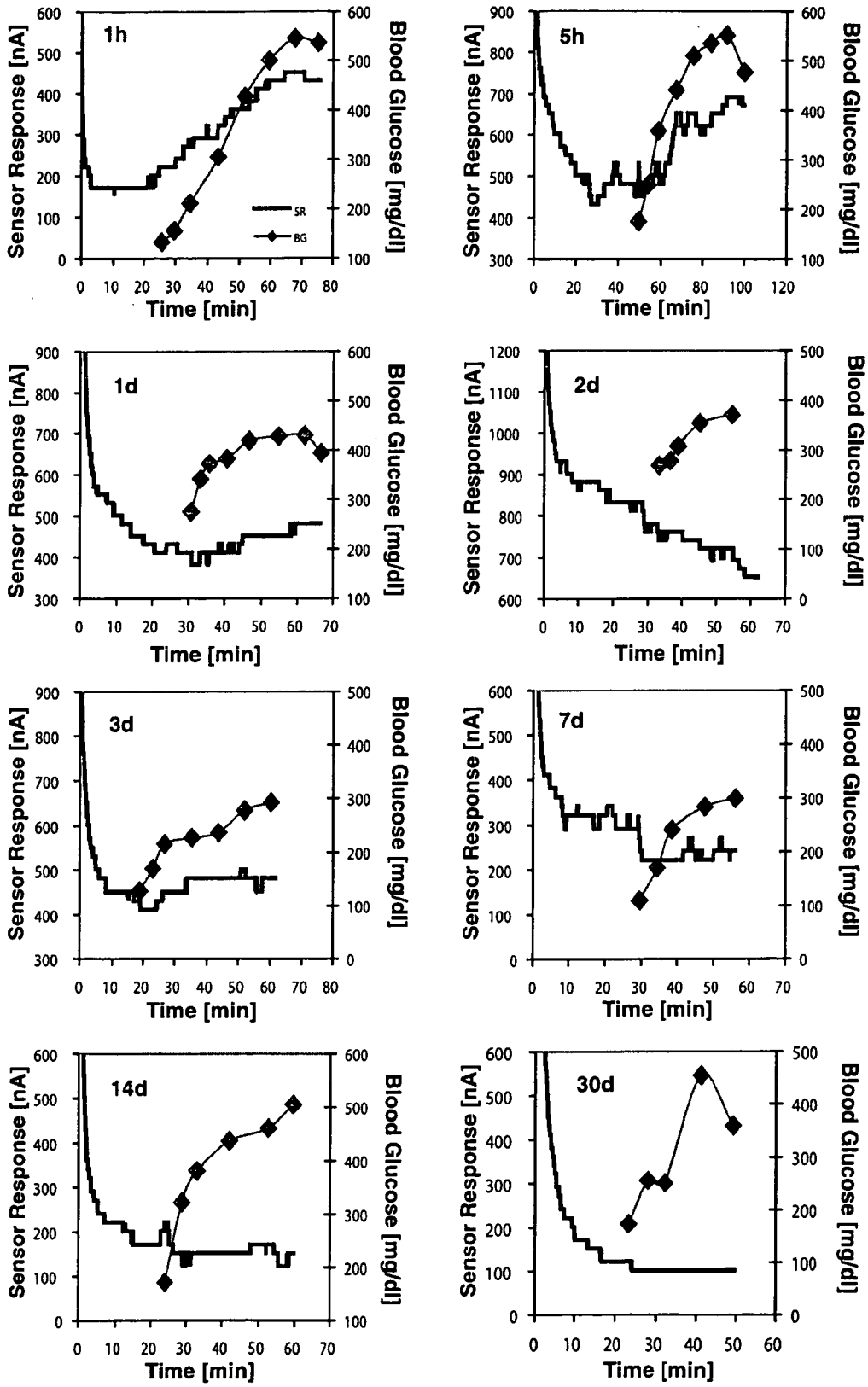

FIG. 2. In vivo function of implantable glucose sensors in ICR mice: sensor function (solid line) and blood glucose levels (line with diamonds) data from a representative ICR mouse. Sensor function is expressed in nA, and blood glucose levels are expressed as mg of glucose/dL of blood.

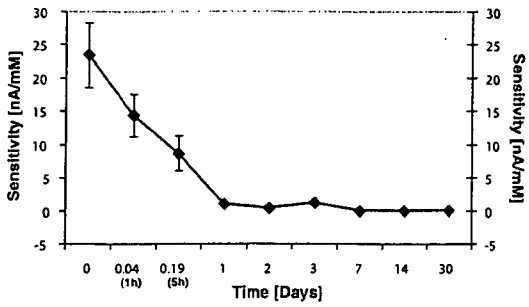

FIG. 3. In vivo functionality of implantable glucose sensors for up to 30 DPI in ICR mice. Data summarize in vivo sensor function of implantable glucose sensors versus time post-implantation in the murine model ($n = 24$ mice). Sensor function is expressed as sensitivity, i.e., nA/m*M* glucose.

excluded from the study because they dislodged the sensor and sensor testing could not be completed.

Acute and chronic tissue responses to implanted glucose sensors

Our analysis of tissue reactions primarily focused around the working electrode (which contains glucose oxidase) and the reference electrode (which lacks glucose oxidase but does release silver chloride); the bridge wire between the electrodes displayed significantly less tissue reaction when compared with the highly tissue-reactive working electrode and

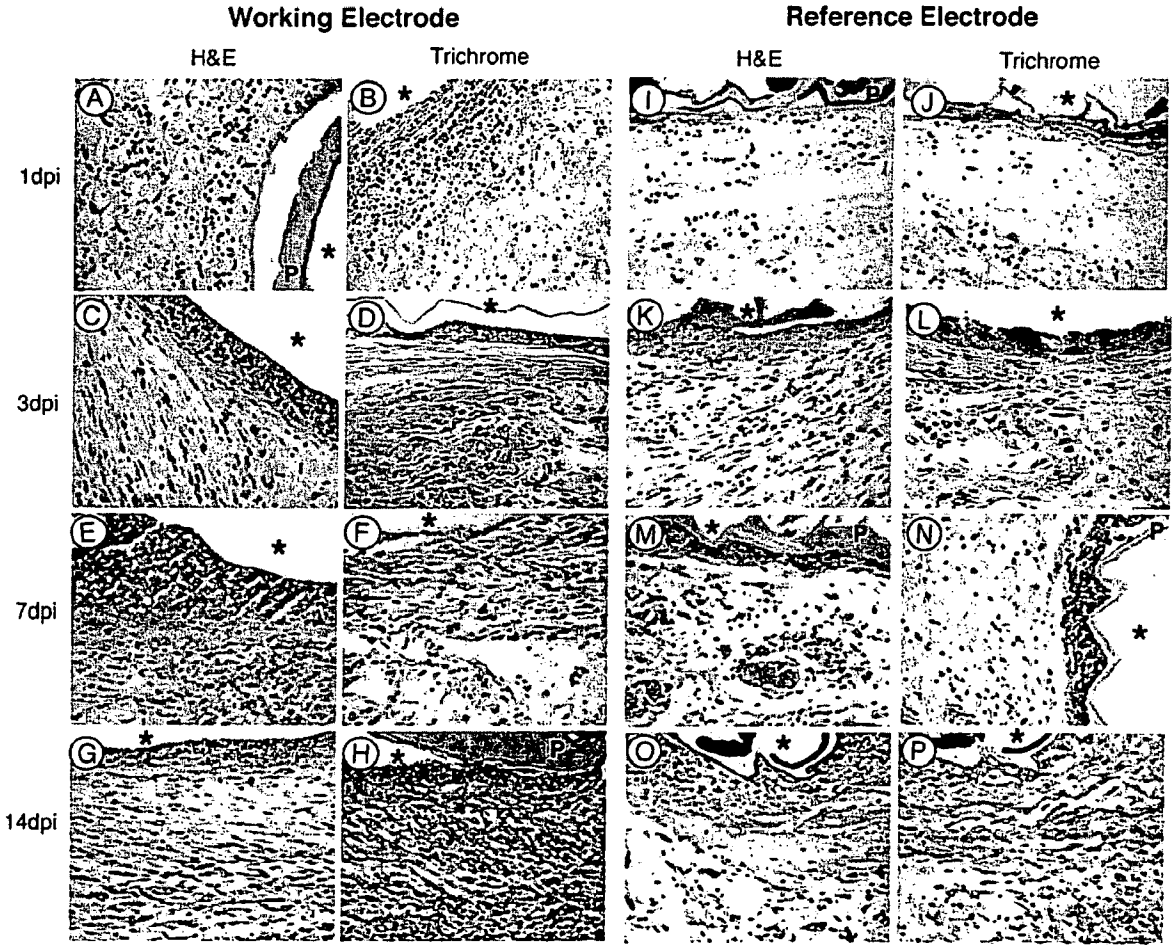

FIG. 4. A–P: Histopathologic evaluation of the tissue reactions induced in ICR mice by implanted glucose sensors. To evaluate tissue reactions at sites of sensor implantation, mouse tissue was obtained from the tissue sites adjacent to the sensors implanted for 1, 3, 7, or 14 DPI. The resulting tissue was processed for histopathologic evaluation as described in Materials and Methods. Both standard hematoxylin and eosin (H&E) and trichrome stainings were used for this study. The original sensor location is designated by (*), and residual sensor coatings are designated by (P).

the moderately tissue-reactive reference electrode.

Histopathology at the working electrode. At day 1 there was a moderate diffuse inflammatory process, characterized by necrosis, edema, fibrin deposition, and the presence of both polymorphonuclear leukocytes (PMNs) and monocytes (Fig. 4A and B). By 3 DPI, the sensor was surrounded by a dense band of inflammatory cells with some necrosis surrounding the implanted sensor (Fig. 4C and D). The inflammatory cells were primarily PMNs and macrophages. Adjacent to the dense inflammatory band, a diffuse region of inflammation characterized by numerous activated macrophages and fibroblasts with occasional lymphocytes was seen (Fig. 4C). Initial collagen deposition was present distal to the band of inflammatory cells surrounding the implanted glucose sensor (Fig. 4D). By days 7 and 14 post-sensor implantation, the tissue reactions were characterized by the presence of large numbers of macrophages and activated fibroblasts surrounding the working electrode (Fig. 4E–H). It was also noted that there was significant neovascularization of the tissue adjoining the working electrode. There was intense giant cell formation particularly around the working electrode as early as 7 DPI (Fig. 5). Trichrome staining of the day 7 and 14 tissue surrounding the working electrode demonstrated the presence of numerous activated fibroblasts associated with growing ribbons of collagen surrounding the working electrode (Fig. 4F and H). By day 30, the working electrode was surrounded by a dense band of inflammatory cells and activated fibroblasts (Fig. 6A and B), intermingled with a dense band of collagen (Fig. 6E and G).

Histopathology at the wire and reference electrode. We observed significantly less inflammatory reaction in the tissue surrounding the reference electrode and the Teflon-coated wire, which joins the working electrode with the reference electrode (Figs. 4 and 6). At 1 DPI, the tissue surrounding the reference electrode had light diffuse inflammation, characterized by the presence of PMNs and monocytes as well as edema and some fibrin deposition (Fig. 4I and J). It was noteworthy that there was relatively little tissue necrosis immediately surrounding the reference electrode, compared with what was seen surrounding the working electrode. At 3 days post-sensor implantation, inflammation characterized by PMNs and macrophages was still present, but was localized close to the surface of the reference electrode (Fig. 4K and L). The inflammatory reactions did not extend significantly into the tissue adjoining the reference electrode (Fig. 4K). Only wispy collagen fibers were seen in the tissue next to the inflammation (Fig. 4L). At days 7 and 14 a low-grade inflammation, characterized by the presence of PMNs and macrophages, continued in close proximity to the surface of the reference electrode (Fig. 4M–P). There was an increased appearance of activated fibroblasts and loose collagen band formation (Fig. 4N and P). Neovascularization was limited and sparse in the tissue surrounding the reference electrode (Fig. 4M and O). By 30 days post-sensor implantation, the inflammatory reactions were still seen in close proximity to the surface of the reference electrode, with an increasingly dense layer of collagen seen bordering the inflammation (Fig. 6D and H). Al-

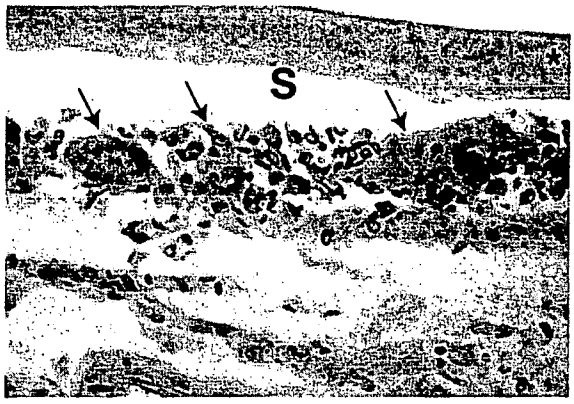

FIG. 5. Demonstration of the presence of giant cells in association with implantable glucose sensors in ICR mice at 7 DPI. To determine if giant cells were seen at sites of sensor implantation, mouse tissue was obtained from the tissue sites adjacent to the sensor implanted for 7 days. The resulting tissue was processed for histopathologic evaluation. Standard hematoxylin and eosin staining was used for this study. The original sensor location is designated by S. Arrows indicate locations of multinucleated giant cells seen in association with the residual sensor polymer coatings (*) of the working electrode of the glucose sensor.

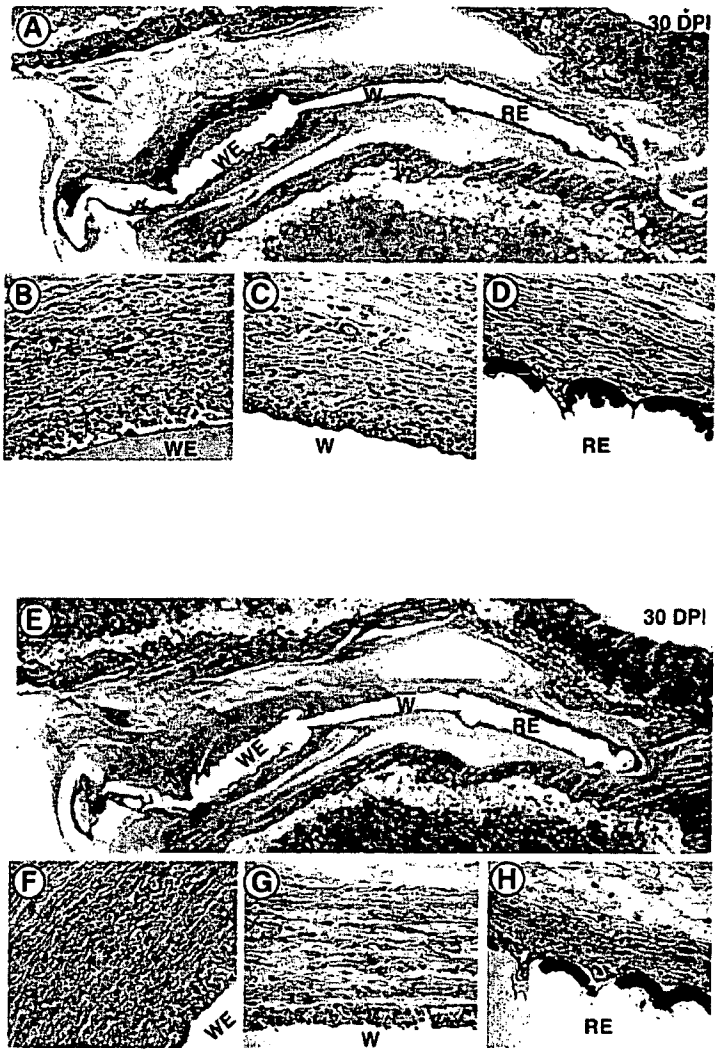

FIG. 6. Demonstration of fibrotic tissue response to implanted glucose sensors at 30 DPI. To evaluate the fibrotic tissue response associated with various segments of the glucose sensor implanted in the mouse model for 30 days, mouse tissue from the sites was obtained and processed for histopathlogic evaluation using hematoxylin and eosin (**A–D**) as well as trichrome (**E–H**) staining. The most intense fibrosis was seen surrounding the working electrode (WE), with marked less fibrosis occurring at the reference electrode (RE), and minimal fibrosis in association with the Teflon-coated wire (W) between the WE and the RE. We speculate that the intense fibrosis associated with the WE is a result of the excessive tissue trauma and inflammation induced by the glucose oxidase by-products (e.g., H_2O_2 and gluconic acid).

though neovascularization was seen distal to the reference electrode it was dramatically less frequent and dense, compared with what was seen at the working electrode.

Quantification of tissue reactions at sensor implantation sites

Tissue reactions at 1–3 DPI were characterized by acute inflammation. By 3–7 DPI, tissue reactions were characterized by the beginning of chronic inflammation and fibrosis. Tissue reactions at days 14–30 post-sensor implantation were characterized by chronic inflammation and fibrosis. The working electrode induced the greatest tissue reaction (Fig. 7). By day 3, both electrodes induced significantly more tissue reaction when compared with the wire (Fig. 7). The working electrode induced significantly more tissue reaction through day 30 of the study (see Fig. 7).

DISCUSSION

The in vivo loss of glucose sensor function is generally considered to be the result of tissue reactions to the implanted glucose sensor, i.e., inflammation, fibrosis, and vessel regression. So far, only small, temporarily placed sensors like the Medtronic (Northridge, CA)[1,2,6] or Therasense-Abbott (Alameda, CA) sensor[3] demonstrate sensor function that is satisfactory to excellent for 3 days. Generally, efforts to ex-

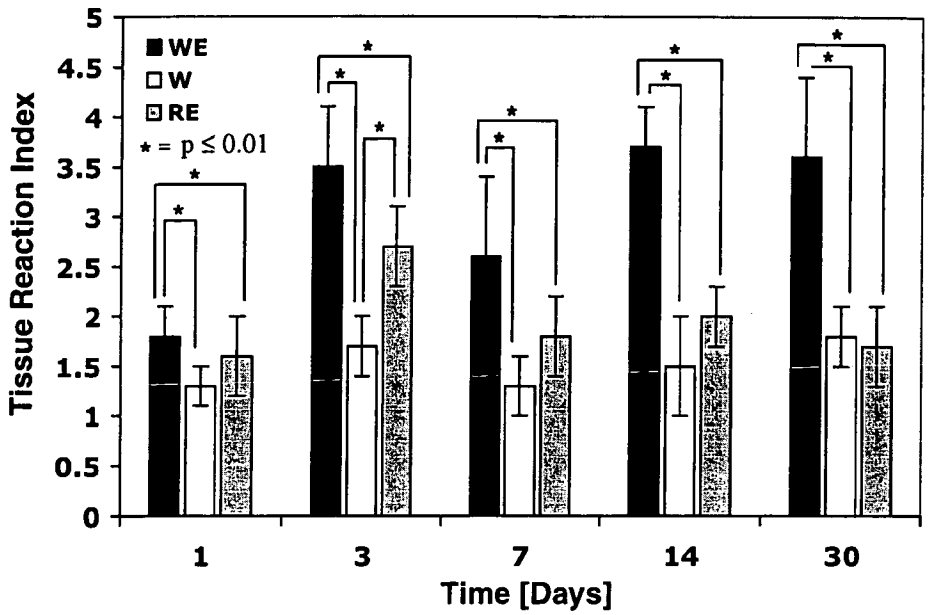

FIG. 7. Quantification of tissue reactions at sensor implantation sites. To compare the tissue responses in various regions of the implanted glucose sensors, i.e., working electrode (WE), wire (W), and reference electrode (RE), at various days post-sensor implantation, hematoxylin and eosin-stained sections of the tissue samples were evaluated using the Tissue Reaction Index described in Materials and Methods. The tissue reactions were scored from 0 (no tissue reactions) to 4 (severe tissue reactions). The resulting data were then analyzed for statistical significance using Student's t test, and significance was considered to occur at $P \leq 0.01$.

tend the in vivo lifespan of implantable glucose sensors have focused on the uses of various sensor coatings, in an effort to "hide or stealth" the sensor from detection and the resulting tissue reactions, but to date these attempts have had limited success. Alternative approaches to extending sensor lifespan have included efforts to suppress inflammatory responses (e.g., dexamethasone doping of the device[9]); and specific stimulation of responses such as angiogenic responses [e.g., vascular endothelial growth factor (VEGF) gene transfer, VEGF infusion, or incorporation of angiogenic membranes around the device[12,15,16]]. Thus, it is clear that without controlling tissue reactions at sites of sensor implantation, glucose sensors have continue to have very short half-lives in vivo, i.e., hours to days, and frequently large numbers of the implanted sensors fail completely once implanted in vivo. Although, the importance of these tissue reactions in sensor function is generally accepted, no systematic research has been performed to determine the cells, factors, and pathways that are responsible for the progressive loss of sensor function observed in vivo. Without this information no rational approach to extending sensor function in vivo can be developed.

The initial subcutaneous implantation of the sensor starts the process by triggering a myriad of vasoactive and pro-inflammatory factors and reactions that affect sensor function.[17,18] Once implanted, biosensors show a shift in their response, requiring frequent re-calibration of the device.[19] This shift is due to initial protein adsorption and cell deposition (inflammatory and tissue cells), making it difficult or impossible for the enzyme glucose oxidase to catalyze the glucose reaction because of limited diffusion of the analyte (i.e., glucose) to the sensor surface.[20] Over time, chronic tissue reactions develop. In most current glucose oxidase-based sensors, glucose and oxygen diffuse to the electrode where the oxidation of glucose produces gluconic acid and hydrogen peroxide, both of which are tissue toxic. Hydrogen peroxide is further oxidized to oxygen, hydrogen, and electrons, likely producing further toxicity from local acidosis. Chronically, the inflammation and tissue destruction ultimately

result in fibrosis with associated vessel regression. These tissue reactions decrease tissue levels of glucose and oxygen within the tissue surrounding the sensor; both of these factors are essential to glucose sensor function in vivo.

We believe that the mouse is a useful animal model to dissect the myriad of mediators and mechanisms that are involved in controlling inflammation, fibrosis, and vessel regression at sites of sensor implantation. In our present studies we demonstrated that sensor implantation in the mouse resulted in both tissue reactions and loss of sensor function that parallel results seen in other animal models (i.e., rats, rabbits, sheep, and dogs).[7] It also demonstrated that the loss of sensor function seen shortly after sensor implantation is associated with the growing tissue inflammation seen in the hours to days after sensor implantation. Our data indicate that the working electrode induces significantly greater inflammatory and fibrotic reactions compared with other segments of the sensor (working electrode and Teflon wire) despite all areas of the electrode having identical surface properties (Nafion coating). We hypothesize that the increased tissue reactivity surrounding the electrode is the result of the release of tissue-toxic factors from the working electrode, including by-products of glucose metabolism by the glucose oxidase (i.e., hydrogen peroxide and gluconic acid). Additionally, it is possible that glucose oxidase leaked from the electrode, because of fracturing of the Nafion layer as a result of calcification of the Nafion.

Our studies also demonstrate that fibroblast recruitment and activation with resulting collagen deposition occur early in the timeline at 3–7 days post-sensor implantation and increase to completely encapsulate the working electrode. Interestingly, at 30 DPI there is still significant inflammation associated with the working electrode, suggesting that despite the collagen deposition tissue injury was ongoing. It is also noteworthy that although neovascularization, as the collagen deposition, was seen early in the tissue reactions 7–14 days post-sensor implantation, increased vessel regression was seen surrounding the working electrode. Clearly, these tissue reactions are dynamic and likely controlled by a variety of factors, cells, and pathways. Understanding the cells and factors involved in tissue reactions such as inflammation, neovascularization, and fibrosis is critical in developing a rational approach to controlling these reactions and enhancing sensor function.

The mouse is a workhorse of modern biomedical research because of the large number of well-characterized mutant and transgenic strains and the wide variety of murine-specific reagents (e.g., antibodies, genes, recombinant proteins, etc.), and technologies (e.g., gene transfer/therapy). The extensive experience in murine-based studies of inflammation, fibrosis, and neovascularization and the vast database of literature on tissue responses in both normal and pathological processes in the mouse make this potentially very important for studies of implantable glucose sensors. In our present studies we demonstrate that the mouse can be used as a simple and effective in vivo model of implantable glucose sensors that displays the same sensor function and tissue reaction seen in other mammals, including humans. The development and validation of a murine model of implantable glucose sensors are critical steps in developing a rational approach to enhancing glucose sensor function in vivo.

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Exhibit 2

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Analysis

Mouse Models of Glucose Sensor Biocompatibility

COL KARL E. FRIEDL, Ph.D.

PERHAPS AS MANY AS ONE IN 10 Americans have permanent medical implants, counting everything from pacemakers and artificial joints to intraocular lenses and dental fillings.[1] In the future, patients with diabetes and soldiers may also have completely self-contained implants that monitor physiological status to provide emergency alerting functions. Eventually, these will provide closed-loop regulation of insulin and controlled release of other drugs. The near-term research efforts are focused on glucose biosensors, and this is a primary goal of the Army's Technologies for Metabolic Monitoring (TMM) research program (www.momrp.org). As shown in the study by Klueh and Kreutzer[2] in this issue of *DT&T* there are some issues specific to chemical detectors, but there is also clearly a much larger community whose research and clinical experience with implants can be leveraged to accelerate the development of implanted glucose sensors.

Only a few years ago, implants to monitor physiological status would have been inconceivable. One problem was subject acceptability, but technological barriers affecting cost, size, power, and telemetry also presented significant problems that have since been largely overcome through engineering solutions. Biocompatibility is now the principal impediment to long-term glucose sensing. The remarkable defenses that thwart sustained sensor performance range from direct cellular attacks on sensor membranes that render the systems inoperable within hours or days, to fibrotic encapsulation and isolation of the sensors that prevent real-time measurement of blood glucose changes. Even stranger then are the long-term successes in some individuals.

In this issue of *DT&T*, Klueh and Kreutzer[2] present an immunologist's perspective on biocompatibility, searching for an improved understanding of the mechanisms of inflammation and wound healing in a systematic approach to regulate and optimize the responses to an implanted glucose sensor. They have revealed an interesting difference in tissue responses to the working and reference electrodes, possibly triggered by the hydrogen peroxide generated in the glucose oxidase reaction. These studies establish the time course of inflammatory responses associated with rapidly diminished functioning of a needle-type glucose oxidase sensor. This expands on the findings of Mang et al.,[3] reported in *DT&T* earlier this year, who observed significant inflammatory responses in rats implanted with glucose oxidase sensors and a progressive reduction in responses with increasing isolation of the chemical probe from the surrounding tissues (using semipermeable membranes and a protective coating). Klueh and Kreutzer[2] also make a case for the mouse as the model for the systematic evaluation of human responses to implantable glucose sensors. They have paid

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special attention to defining and minimizing variability produced by surgical technique, inadvertent infection, baseline status of the animals at time of implantation, and physical and chemical surface properties of the devices. This new work may be the first reported study of glucose sensors in a mouse.

Why the mouse? Worms, fruit flies, and zebra fish—all part of the current inventory of test species with completely defined genomes—are obviously not suitable for glucose sensor compatibility testing, but mice, despite their small size, present many advantages including the extensive genetic characterization. Previous studies on glucose sensors have generally used dogs and rats as the model systems. Companion animals (dogs) are no longer suitable as subjects of invasive medical experimentation, and the TMM program would be unlikely to consider new research involving this species. Rats have the advantage of size, but have not been nearly as well characterized as mice, nor have hamsters or rabbits. A large segment of the research world has already concentrated on the mouse, providing a research tool set that is unparalleled in any other species, with at least 40 well-studied inbred strains, along with their reproducible derivatives, mutants, transgenics, etc. An entire laboratory, the Jackson Laboratory in Bar Harbor, ME, is dedicated to the development of the mouse as the genetic model of human biology (www.informatics.jax.org; www.complextrait.org). (And, in fact, they now desperately need new physiological monitoring tools that will help define the phenome; an implantable glucose monitor for mice would itself be useful in diabetes research.[4])

Response differences between the rat and the mouse also tend to favor the mouse as the better indicator of what will occur in the human. A side-by-side comparison of responses to implanted expanded polytetrafluoroethylene (e-PTFE) (Gore-Tex®, W.L. Gore, Elkton, MD) showed that angiogenic responses were similar between the mouse and rat, but the rat had a greater inflammatory response and tended to form a thicker capsule around the implanted material.[5] Another study compared rat and human responses to subcutaneous polyamide probes. Over 8 days, there was a gradual improvement in microdialysate glucose in the human implants suggesting a healthy angiogenic response, while in the rat implants, glucose progressively decreased and lactate increased as a more aggressive foreign body response occurred.[6]

Other biocompatibility studies with mice have begun to explore the use of genomics. Genomics can easily become a giant "resource sponge" that requires a major long-term commitment to realize any return on the investment. What will it cost, who is going to support this, and what are the payoffs that will convince the research community including both peer reviewers and program managers? Too often it appears to be a knee jerk response as a trendy state-of-the-art technique applied to solve smaller problems that may actually lend themselves to more pedestrian and cost-efficient approaches. But, maybe the diabetes technology community can ride the coat tails of other biocompatibility efforts. In an elegant set of gene activation studies, Schwartz et al.[7] tested ePFTE implants that had been coated with serum-free conditioned medium from a keratinocyte cell line; this provides a "pro-vascular" protein matrix to stimulate greater angiogenic responses and decrease fibrous encapsulation. These studies demonstrated a variety of differences in gene activation between the modified and unmodified ePFTE implants, and they defined the time course.[7] As this work is expanded to other materials, these data will provide a basis for comparison of the healing responses to new implant materials and to biosensors.

Genomic studies could help define the precise factors controlling inflammation and healing, leading to identification of optimal strategies to manage implants. As an example, vascular endothelial growth factor (VEGF) has been explored in optimizing tissue angiogenic responses: Klueh et al.[8] tested the value of local VEGF secretion through genetic overexpression in the chick embryo model; Ward[9] has shown the value of VEGF slow release to sensor responsiveness in rats; and recently Peattie et al.[10] demonstrated a VEGF slow release hydrogel system in mice. Genetic studies with mice providing high and low tissue responses could provide a more systematic approach to identifying the factors behind the optimal in-

flammatory and vascular response. Perhaps there is a more fundamental factor such as hypoxia inducible factor-1a behind VEGF release and related responses, or an early stage interleukin-6 suppression of other inflammatory responses. Better-defined markers of inflammatory responses would improve tools for the evaluation of materials, surface properties, chemical coatings, cytokine-doped materials, etc. Comparison of responses in the peritoneum, fat, muscle, and subcutaneous sites could begin to explain local environmental interactions that determine relative success of implants, including the better angiogenic and neovascular responses in fat tissue. Responses in other strains, including diabetic and obese mouse strains, may identify important differences in healing and inflammation—a potentially serious confounder when glucose implants start going into patients with diabetes. The diabetes technology community should consider the recommendation of Klueh and Kreutzer[2] and aggressively exploit well-developed murine models.

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Exhibit 3

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Continuous Glucose Monitoring in Normal Mice and Mice with Prediabetes and Diabetes

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ABSTRACT

It is well established that the key to minimizing diabetes-associated complications, in both type 1 and type 2 diabetes, is tight regulation of blood glucose levels. Currently the major approach to regulating blood glucose levels in patients with diabetes relies on external blood glucose monitors. However, poor patient compliance usually results in limited insights into the dynamic range of blood glucose levels (i.e., hyperglycemia vs. hypoglycemia), and inadequate prediction and control of blood glucose levels in these patients. Implantable glucose sensors hold promise for controlling blood glucose levels, but currently these sensors have only limited in vivo life span. Recently we have developed an extremely robust murine model for implantable glucose sensors. In the present study, we have extended this model by developing a complete system for real-time continuous glucose monitoring in normal mice and mice with prediabetes and diabetes (type 1). These studies demonstrated that (1) glucose sensors can be implanted and maintained subcutaneously in the mice; (2) continuous glucose sensor data can be obtained for at least 5 days; and (3) subcutaneous blood glucose sensing paralleled blood glucose levels in normal mice and mice with prediabetes and diabetes. Subcutaneous blood glucose sensing also successfully tracked changes in blood glucose levels induced in the mice with diabetes by administration of oral glucose or insulin. These results mirror the results for subcutaneous blood glucose sensing seen in both normal subjects and patients with diabetes, and therefore validate both our continuous glucose monitoring system in the mouse, and the use of the mouse as a model for implantable glucose sensing in vivo.

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INTRODUCTION

DIABETES IS A CHRONIC PROGRESSIVE DISEASE, which is growing at epidemic rates both in the United States and in the world. Diabetes has tremendous costs, not only in human suffering, but also economically, with U.S. costs of diabetes estimated at over 150 billion dollars annually. The key factor in preventing the devastating complications and death associated with diabetes is the close monitoring of blood glucose levels.[1–3] This is generally accomplished by episodic (approximately four times daily) monitoring with disposable test strips, typically using capillary blood obtained by fingerstick. Unfortunately, because of the pain and inconvenience associated with "finger sticking," patient compliance is often poor. Even with good patient compliance and regular blood glucose testing, blood glucose swings or "excursions" are often undetected and directly contribute to the complications and deaths associated with diabetes. For example, initial continuous glucose monitoring (CGM) of patients with type 1 diabetes has shown that glucose concentrations are only within a target range of 4–10 mmol/L for about 35% of the time.[4] Clearly, there is a critical need for a method that would allow long-term continuous blood glucose monitoring in vivo.

One solution to this problem is implantable subcutaneous glucose sensors. Although implantable glucose sensors have existed for over 30 years, relatively little progress has been made in their commercialization. The development of a successful implantable glucose sensor requires controlling and combining three critical aspects: (1) sensors, (2) telemetry, and (3) controlling tissue reactions at sites of sensor implantation. Although sensor design and telemetry have progressed, there has been little progress in controlling tissue reactions that limit sensor function and lifespan to hours or days. These tissue reactions include the triad of inflammation, fibrosis, and vessel regression. Unfortunately, little is known about the mediators and mechanisms that control these tissue responses to implantable glucose sensors. This is mainly because of the lack of a robust animal model that would allow mechanistic insights into sensor-induced tissue responses.

To fill this gap, we have recently developed the first murine model of implantable glucose sensors, based on a needle-type hydrogen peroxide-detecting glucose sensor.[3,5] Using this model, we demonstrated that: (1) sensor function was dramatically reduced within hours and totally lost within days post-implantation and (2) loss of sensor function was correlated with tissue reactions at the sites of sensor implantation. In the present study, we have extended this model by developing a complete system for real-time CGM in normal mice and mice with prediabetes and type 1 (juvenile) diabetes [non-obese diabetic (NOD)], based on a Wired Enzyme™ [TheraSense (now Abbott Diabetes Care), Alameda, CA] glucose sensor. The NOD mouse is an autoimmune model of type 1 diabetes, and has been used extensively to investigate not only the immunopathogenesis of type 1 diabetes, but also a wide variety of potential treatment approaches for type 1 diabetes in humans.[6,7] Utilizing these NOD mice we demonstrated that (1) glucose sensors can be implanted and maintained subcutaneously in mice and (2) continuous glucose sensor (CGS) data can be obtained for at least 5 days. These studies demonstrated that CGM in the NOD mouse is an effective model that displays sensor function similar to that seen in humans. Most importantly, this robust spontaneous animal model should prove helpful in unraveling the mediators and mechanisms that control tissue reactions and sensor function in vivo. Understanding the factors that control the loss of glucose sensor function in vivo will provide the foundations for rational approaches to developing CGM with extended function and lifespan.

MATERIALS AND METHODS

System for CGM in the mouse

A schematic diagram of the CGM system used in the mouse model is shown in Figure 1. For CGM, glucose sensor leads were connected via a commutator to a potentiostat (Petite Am-

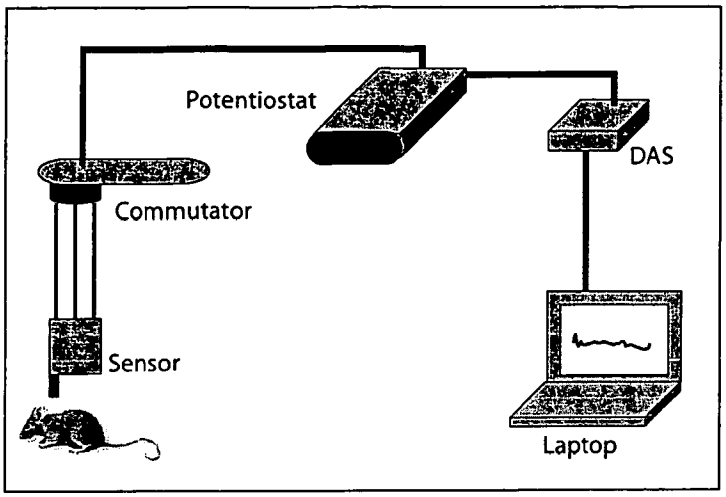

FIG. 1. Schematic diagram of the CGM system used in the mouse model. This CGM system includes: (1) an amperometric sensor developed by Abbott Diabetes Care[9]; (2) a commutator to allow free roaming of the mouse; (3) a potentiostat; (4) a data acquisition system (DAS); and (5) a standard laptop computer for continuous data gathering, analysis, and display.

pere, Bioanalytical Systems, Inc., West Lafayette, IN), which maintains the glucose-sensing working electrode at a potential of +40 mV versus the Ag/AgCl reference electrode. The potentiostat was then connected to a data acquisition system (Abbott Diabetes Care), which captured and sent real-time glucose-derived current data (typically 1–30 nA) from the potentiostat to a laptop computer, at 60-s intervals. Reference blood glucose concentrations were obtained from the transected distal tail of the mice using a standard FreeStyle® Blood Glucose Monitor (Abbott Diabetes Care), and were logged into the continuous data system, as was any glucose or insulin administration.

Normal mice and mice with diabetes

Female NOD/LtJ mice (5–7 weeks old) and C57BL/6J:strain 000664 (5–7 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME). The NOD/LtJ mice initially did not have diabetes, but progressively developed type 1 diabetes by week 17–40 of life. The C57BL/6J:strain 000664 mice were matched normal controls for the NOD/LtJ mice. All animals were housed in the Animal Facility at the University of Connecticut School of Medicine (Farmington, CT) prior to sensor implantation and subsequent sensor testing. Mice were housed in our laboratory for all testing periods of up to 7 days. All work with mice was approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center (Farmington).

Monitoring of diabetes onset in NOD/LtJ mice

To monitor the onset and severity of type 1 diabetes in the NOD/LtJ mice, blood was obtained from the tail of the mice, and blood glucose levels were determined using the external glucometer. Blood glucose measurements were conducted weekly in the mice. Mice were considered to have diabetes if blood glucose levels were above 250 mg/dL for two sequential blood glucose tests. Mice were considered to have prediabetes when mouse blood glucose levels were in the range between 150 and 250 mg/dL for two sequential blood glucose tests. NOD mice were considered normal when their blood glucose levels were below 150 mg/dL at any given time. All glucose tests were conducted in the non-fasting state.

Glucose sensors

All glucose sensors used for these studies were fabricated at Abbott Diabetes Care. These glucose sensors are identical to those used in the FreeStyle Navigator™ CGM system, and are based on the Wired Enzyme™ sensing technology as previously described,[8,9] which has demonstrated close correlation between subcutaneous and venous blood glucose values in human subjects[9] (Fig. 2A and B). In order to protect the bare sensors from shorting out, sensors were covered in electrical moisture sealant (3M Corp., Minneapolis, MN). All sensors were pretested in vitro using a standard dextrose solution (100 mg/dL dextrose in a phosphate-

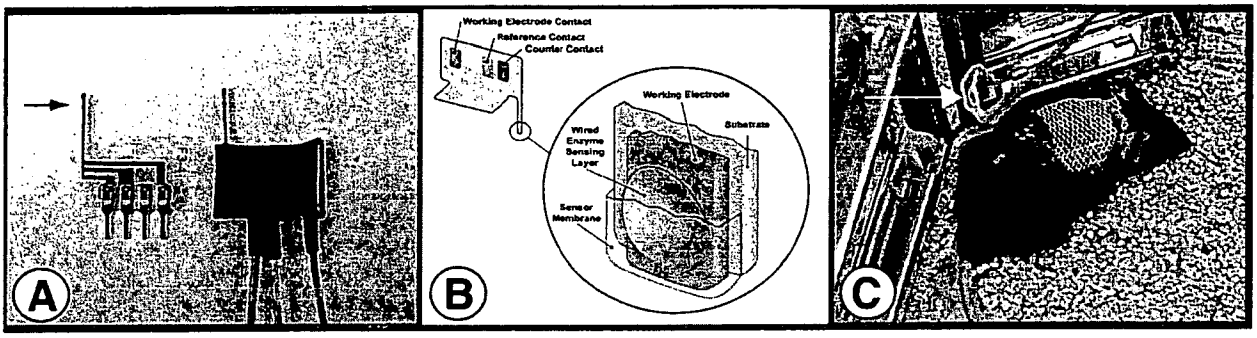

FIG. 2. Implantable glucose sensor used for continuous blood glucose sensing in mice: the Abbott (formerly TheraSense) glucose sensor with (right) and without (left) protective electronic coating, with the arrow indicating the sensing tip (**A**); and diagrammatic representation of the Abbott glucose sensor by Feldman et al.[9] (**B**). (**C**) C57BL/6J control mouse with implanted glucose sensor and cable (arrow) connecting to the commutator for continuous blood glucose measurement.

buffered solution, pH 7.2) prior to implantation (see below for implantation procedure).

Glucose sensor implantation in the mouse

The glucose sensors described above were implanted in the mice as previously described by our laboratory[3] with slight modification. Briefly, 150 μL of sterile, pyrogen-free, 0.9% NaCl was injected subcutaneously in the interscapular shaved area of the mouse to provide an "implantation pocket." Next, a small incision was made using a 21-gauge needle, and the sensor tip was carefully inserted into the opening. The electrical moisture sealant, covering the external parts of the sensor (see Fig. 2A), was glued to the mouse skin using a protective coating (New-Skin First Aid and Antiseptic Liquid Bandage, Medtech Corp., Jackson, WY). A nylon mesh was placed on top of the electrical moisture sealant in order to secure the sensor from dislodging by the mouse (Fig. 2C). Mice were kept under 1% isoflurane anesthesia until the protective coating dried. Mice were housed individually to prevent dislodging of the sensor from aggressive behavior between the mice.

CGM in vivo

For CGM, glucose sensors were implanted as described above. Immediately after sensor implantation, CGM was initiated using the system described in Figure 1. Glucose-derived current data were obtained at 60-s intervals. Blood glucose reference measurements were obtained periodically over the 5–7-day implantation period, using blood obtained from the tail vein (~0.3 μL) and a FreeStyle Blood Glucose Monitor. These reference blood measurements were used to calibrate the current data from the continuous subcutaneous sensor, as follows. A single, optimum sensitivity (expressed in current/unit glucose concentration) was calculated for each individual sensor, such that application of this sensitivity resulted in the lowest possible mean absolute relative deviation (MARD), compared with the capillary reference values. This is a retrospective calibration process, not able to provide real-time glucose values, but useful for evaluating sensor accuracy. Sensor accuracy was further evaluated by calculation of the MARD for each sensor.

Mice with diabetes (blood glucose measurements >300 mg/dL) were administered a combination of intermediate (NPH human insulin) and long-lasting (Lente® human insulin) (Eli Lilly and Co., Indianapolis, IN) insulin (1.5–3 units/kg, s.c.). Dextrose (0.5 g/mL; Sigma, St. Louis, MO) was made available orally to the mice whenever glucose levels fell below 50 mg/dL. We utilized oral dextrose, rather than intraperitoneal injection, because we found that the oral method was less stressful to the free roaming mice. For CGM, the following groups of mice were evaluated: (1) normal mice (C57BL/6J); (2) normal NOD mice (i.e., normal blood glucose levels); (3) NOD mice with pre-

diabetes (i.e., elevated blood glucose levels); and (4) NOD mice with diabetes.

RESULTS

CGM system for blood glucose sensing in mice using an implantable glucose sensor

Using the design presented in Figure 1, the CGM system (Fig. 3) was validated in vitro using varying concentrations of glucose. In vitro sensor sensitivity generally ranged from 0.04 to 0.09 nA/(mg/dL) glucose. For in vivo studies, sensors were implanted into the mice, and the sensors were well tolerated (Fig. 2C). We found that using the netting to secure the sensors was important in preventing removal of the sensors (Fig. 2C). The commutator allowed free roaming without entanglement of the mice in the wire or removal of the sensor. We also found that using a round containment device (i.e., a metabolic cage with a bottomless plastic bucket on top) allowed the mice continuous access to food and water, as well as free roaming. The bottomless plastic bucket eliminated the need for the lid on top of the metabolic cage and preventing the mouse from escaping.

CGM in normal mice (C57BL/6J)

We began our studies by first evaluating CGM in normal mice (C57BL/6J) that are matched controls for the NOD mice with diabetes. As can be seen in Figure 4, glucose sensing closely tracked with the blood glucose levels in the normal mice over a 5-day period. Specifically, blood glucose levels in the C57BL/6J mice generally ranged from 100 to 200 mg of glucose/dL of blood, with CGS current ranging from 4 to 20 nA (n = 6). The two sensors shown have sensitivities of 0.061 and 0.071 nA/mg/dL and MARD values of 9.9% and 13.9%. These values compare with a value of 10.9% found in a separate study (110 implanted sensors) of Navigator usage in humans without diabetes. With these baseline data we next evaluated CGS in normal mice and NOD mice with prediabetes and diabetes.

CGM in NOD mice

CGM in normal NOD mice. In phase 1 (<20 weeks of age) NOD mice did not have diabetes, with normal blood glucose levels. Evaluation of our glucose sensing system in these NOD mice without diabetes produced results similar

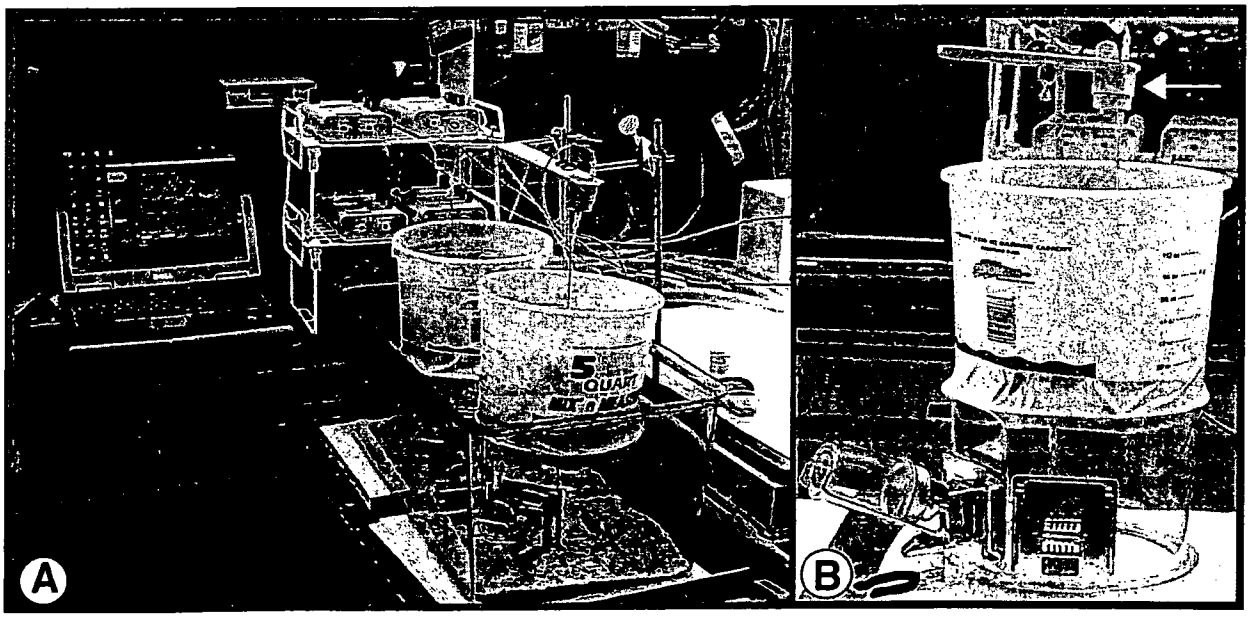

FIG. 3. System for continuous monitoring of blood glucose levels in mice: the CGM system used in mouse models (as represented in Fig. 1) (A) and showing the mouse containment system with food and water dispensers and the commutator (arrow) (B).

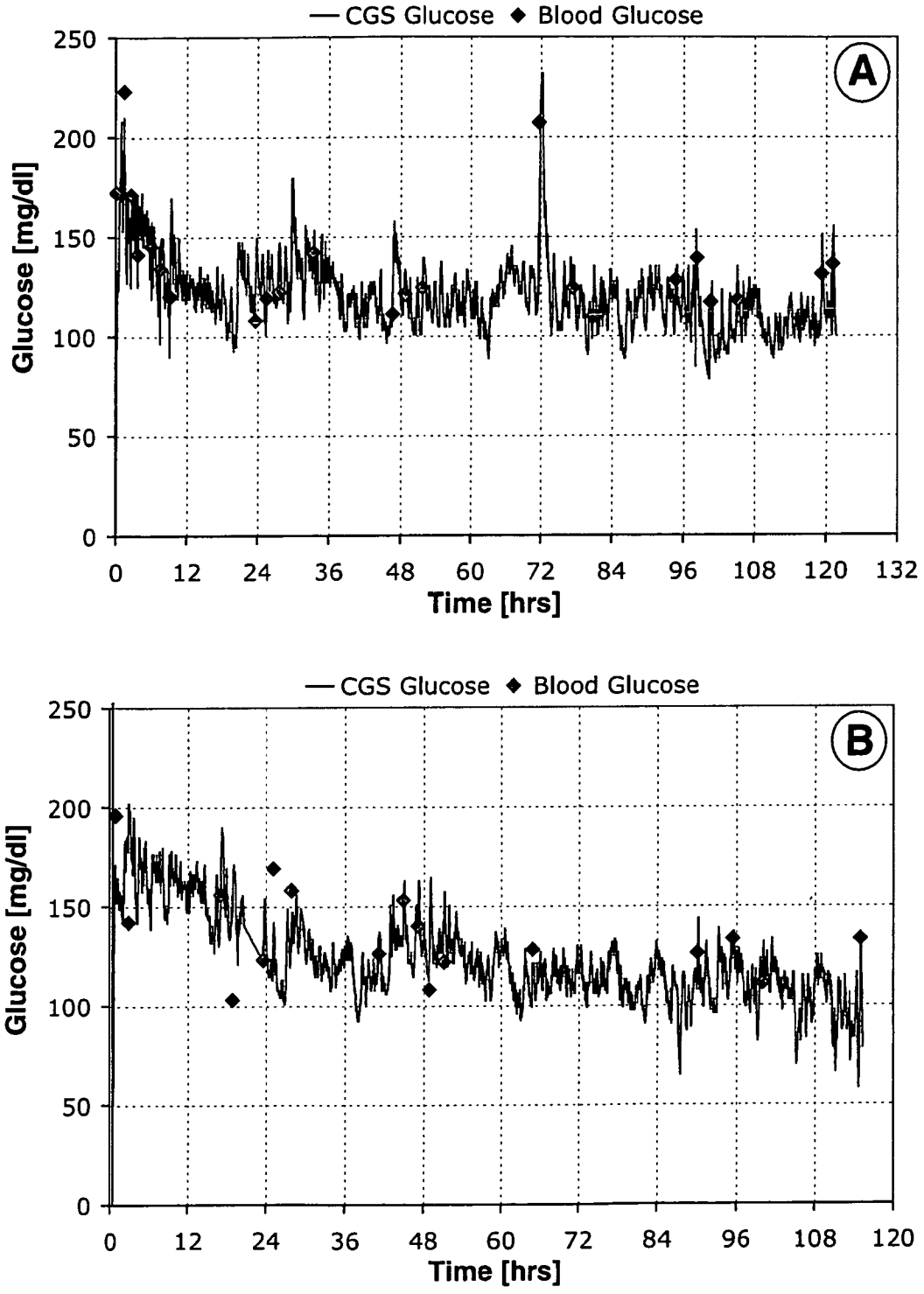

FIG. 4. Calibrated continuous glucose data for sensors implanted in normal C57BL/6J mice. (A and B) Representative CGM results for two normal C57BL/6J mice. CGS and blood glucose levels were evaluated over a 5-day period after sensor implantation (CGS glucose = solid blue line; blood glucose levels using a FreeStyle glucose monitor = red diamonds). Six normal C57BL/6J mice were used in this study.

to those in the C57BL/6J normal mice (Fig. 5). Specifically, blood glucose levels ranged from 100 to 200 mg/dL of blood, with CGS current ranging from 4 to 20 nA (n = 6). For the sensor shown, sensitivity was 0.072 nA/m*M*, and the MARD was 12.1%. Thus, our CGM system appears to accurately track blood glucose levels in normal NOD mice over a 5-day period, with accuracy similar to that seen in normal humans.

CGM in NOD mice with prediabetes. Our system also closely tracked blood glucose levels in the NOD mice with prediabetes (Fig. 6). Blood glucose levels displayed wide glucose excursions (100–300 mg/dL), consistent with the general definition of prediabetes. CGS current closely tracked blood glucose levels in the NOD mice with prediabetes (Fig. 6, sensitivity = 0.082 nA/m*M*, MARD = 12.2%). These data demonstrate the effectiveness of both the Abbott sensor and our CGM system in successfully tracking blood glucose values in mice with prediabetes.

CGM in NOD mice with diabetes. NOD mice with diabetes displayed wide swings in blood glucose levels, ranging from 20 mg/dL to 500 mg/dL (Fig. 7), characteristic of brittle diabetes in humans. We lowered blood glucose levels in these mice with subcutaneous insulin injections, and oral glucose elevated blood glucose levels (Fig. 7). Glucose sensing closely paralleled blood glucose levels under all of the glycemic conditions, i.e., hypoglycemic and hyperglycemic, as well as after use of insulin and oral glucose. Because of the wide glucose excursions in the NOD mice with diabetes the CGS current ranged from 4 to 80 nA over the 5 days post-sensor implantation. For the sensor shown, the sensitivity was 0.045 nA/(mg/dL), and the MARD was 9.3%. These data closely parallel glucose sensing data, obtained with the Navigator im-

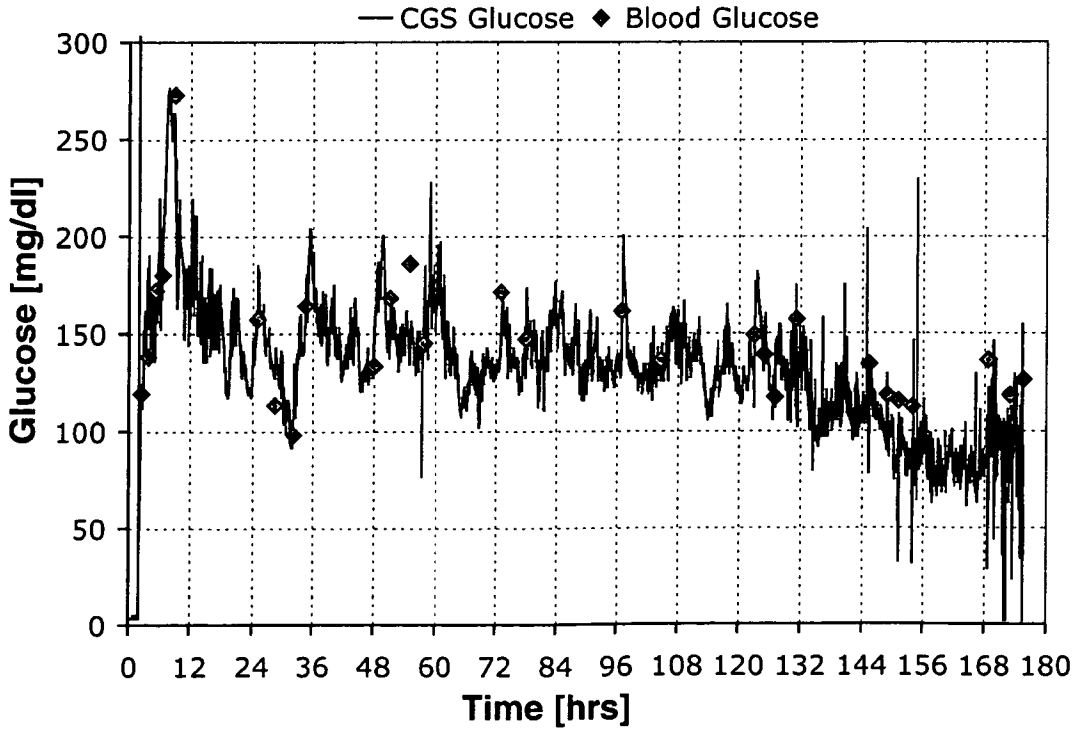

FIG. 5. Calibrated continuous glucose data for sensors implanted in normal NOD mice. Representative results are shown for CGM in normal NOD mice, evaluated over a 7-day period after sensor implantation (CGS glucose = solid blue line; blood glucose levels using a FreeStyle glucose monitor = red diamonds). Six normal NOD mice were used in this study.

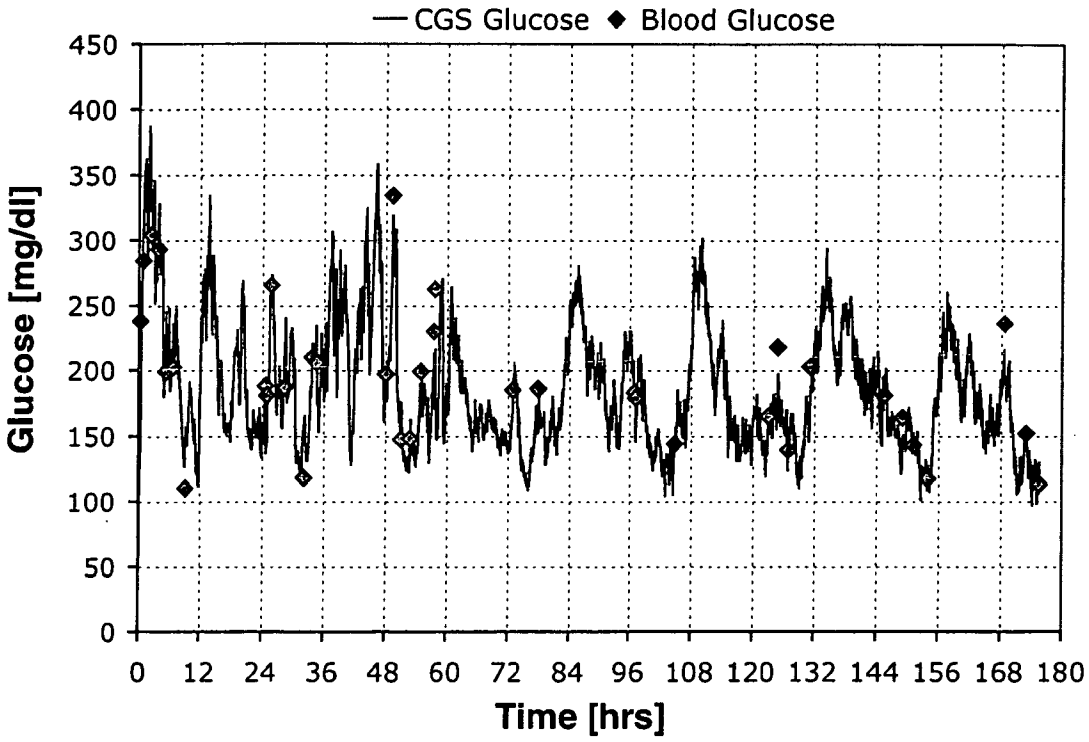

FIG. 6. Calibrated continuous glucose data for sensors implanted in NOD mice with prediabetes. Representative results are shown for CGM in NOD mice with prediabetes, evaluated over a 7-day period after sensor implantation (CGS glucose = solid blue line; blood glucose levels using a FreeStyle Glucose monitor = red diamonds). Five NOD mice with prediabetes were used in this study.

plantable glucose sensor implanted in patients with diabetes.[9]

DISCUSSION

These studies clearly demonstrate that our CGM system can successfully monitor blood glucose levels in normal mice and NOD mice with prediabetes and diabetes, and that these data parallel data obtained with the Navigator sensors in subjects with and without diabetes. Technology that allows long-term continuous monitoring of blood glucose could revolutionize the treatment of both type 1 and type 2 diabetes. Development of an implantable glucose sensor capable of long-term CGM holds tremendous potential for the effective management of diabetes.

The concept of an implantable sensor has existed for over 30 years, but there has only been limited success in developing a commercially viable implantable glucose sensor that lasts consistently for more than a few hours or days.[9–14] Even experimental glucose sensors have seen variable levels of success in functionality and lifespan in vivo.[15–17] While it is generally accepted that the loss of function is associated with sensor-induced tissue reactions, i.e., inflammation and fibrosis, little is known about the mediators and mechanisms that are responsible for this loss of glucose sensor function in vivo. This lack of information is in part due to (1) the limited number of in vivo studies, (2) lack of significant characterization of tissue reactions and correlation with sensor functions, and (3) the lack of a robust animal model that provides the foundation for a rational approach to enhance sensor. Traditional in vivo sensor models (e.g., rabbits, rats, dogs, and cats) lack the genetics and reagents (antibodies, genes, etc.) to develop mechanistic insights into the causes of the loss of sensor function in vivo. It is clear that identifying the specific cells, factors, and pathways that control the tissue reactions and sensor function is

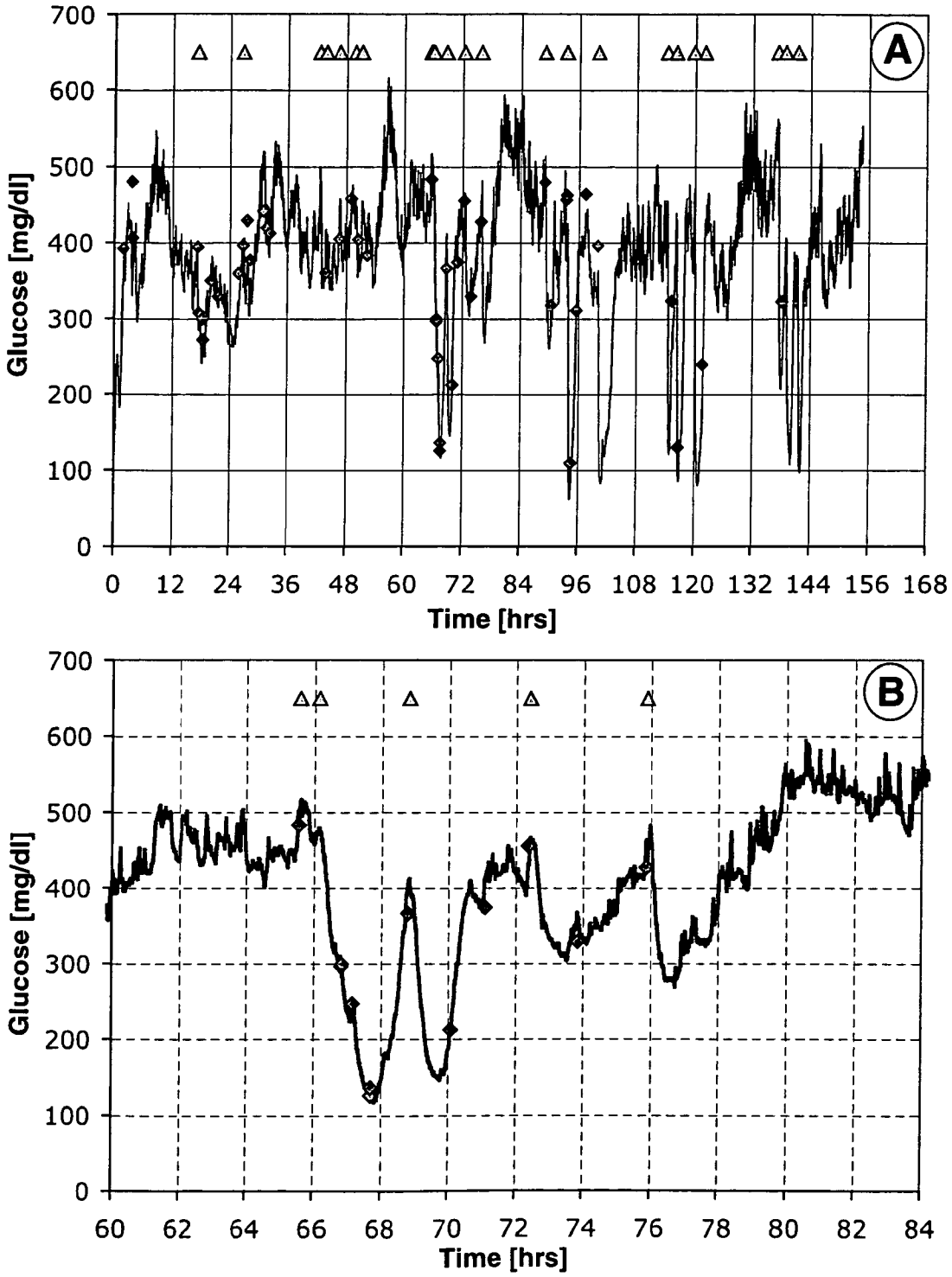

FIG. 7. Calibrated continuous glucose data for sensors implanted in NOD mice with diabetes. (A) Representative results are shown for CGM in NOD mice with diabetes, evaluated over a 6-day period post-sensor implantation (CGS glucose = solid blue line; blood glucose levels using a Freestyle Glucose monitor = red diamonds; insulin administration = triangles). (B) A magnified view of the data from (A) for the period 62–84 h after implantation, showing the sensor response to insulin injections. Ten NOD mice with diabetes were used in this study.

critical to developing a rational approach to controlling these reactions and enhancing the sensitivity and lifespan of sensors in vivo. An animal model can provide the information needed to develop a rationale approach to designing a biocompatible glucose sensor with extended in vivo lifespan.

Our laboratory recently developed and validated a murine model for in vivo glucose sensor testing, which can be utilized to define specific mechanisms of sensor degradation.[3] In the present study, we have extended this model by developing a complete system for real-time CGM in normal mice and (NOD) mice with prediabetes and type 1 (juvenile) diabetes. The NOD mouse is an autoimmune model of type 1 diabetes, and has been used extensively to investigate not only the immunopathogenesis of type 1 diabetes but also a wide variety of possible treatments approaches for type 1 diabetes in humans.[6,7] The first reports on NOD mice with diabetes appeared nearly 20 years ago, and since then this strain has evolved into the major animal model for type 1 diabetes.[6,7] Additionally, mouse models of type 2 diabetes (e.g., NONcNZO10/LtJ, NZO mice) have also been developed, and are seeing increasing interest because of the explosion of type 2 diabetes in the United States and around the world. The NOD mice are considered an excellent model for human type 1 diabetes since they share many of the same characteristics with human diabetes, including progressive onset. Additionally, NOD mice have pancreatic reactive antibodies and autoreactive T cells, and genetic linkages similar to those seen in human diabetes.[6] The NOD mouse has provided and continues to provide important fundamental insights into the underlying mediators and mechanisms associated with type 1 diabetes and its management. In addition, the NZO mouse, a model for type 2 diabetes, shows promise for developing a better understanding of the pathogenesis and management of that disease. Therefore, the use of NOD mice, and NZO mice in the future, will build on our knowledge of, and treatment approaches for, human diabetes.

In our present study, we evaluated CGM in a murine model of type 1 diabetes, i.e., the NOD mouse, which develops type 1 diabetes spontaneously. Our studies utilizing these NOD mice demonstrated that: (1) glucose sensors can be implanted and maintained subcutaneously in mice; (2) CGS data can be obtained for at least 5 days; and (3) using the Navigator sensor, blood glucose levels paralleled glucose sensing in normal mice and mice with diabetes. These studies demonstrated that the NOD mouse is an effective model that displays sensor function similar to that seen in humans.[9] Most importantly, this animal model should prove helpful in unraveling the mediators and mechanisms that control tissue reactions and sensor function in vivo. The ability of the CGS to closely track blood glucose levels is critical since it is a major step in the development of any closed loop system. Thus, we believe that the development of this mouse model of continuous blood glucose sensing will not only provide insights into the function of glucose sensors in vivo, but will also help to advance the development of closed loop systems for the future.

ACKNOWLEDGMENTS

This work was supported by the U.S. Army Technologies for Metabolic Monitoring Research Program, Fort Detrick, MD (USAMRMC Grant Award 03169004), Abbott Diabetes Care, Alameda, CA, and the BEACON Foundation, Hartford, CT.

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Exhibit 4

DIABETES TECHNOLOGY & THERAPEUTICS
Volume 8, Number 3, 2006

Analysis

Overcoming the "Valley of Death": Mouse Models to Accelerate Translational Research

COL KARL E. FRIEDL, Ph.D.

THE RESULTS OF WELL-DESIGNED research projects inevitably suggest more experiments. In contrast, problem solving and product development require a narrowing of available options to produce a best solution. Between these two fundamentally different cultures of researchers and developers is the so-called "valley of death," where discoveries fall into a technology purgatory instead of smoothly transforming into new building blocks accessible to the problem solvers.[1] This cultural divide is reinforced by the existing organizational structures that want to fund only the most innovative mechanistically based projects and investors who want reasonable feet-on-the-ground indicators of likely success. So it is disappointing but not too surprising that discoveries from diabetes research on the benefits of tight glucose monitoring and regulation, and ideas on implantable systems to do the monitoring that will also lead to the artificial pancreas, have not moved faster into solutions for the diabetes community. From a traditional researcher perspective, we will always need refinements and more study, and clues to new solutions may not get the needed push out of the tech base. Many researchers are even surprised and frustrated that developers are not readily plucking their great discoveries out of the tech base. This leap to applications requires early prototyping and proofs of concept. New models save time and money in testing new treatments and technologies, and they can help researchers push their discoveries out by providing adequate demonstration of the relevant applications. In this issue of *DT&T*, such a model is presented: a free-ranging mouse model with continuous glucose monitoring.[2]

Klueh et al. have followed up on their previous report of the mouse as a model for foreign body tissue responses to implanted glucose sensors.[3] In the present paper[2] they have perfected their implantation techniques and other methods that improve reliability of the sensor system. Co-investigators from Abbott Diabetes Care (Alameda, CA) miniaturized the FreeStyle® Navigator™ continuous glucose monitor to produce an effective device for use in relatively unencumbered free-ranging mice. This robust animal model provided continuous glucose measures that closely parallel tail vein glucose measurements in healthy mice and mice with prediabetes and diabetes. The non-obese diabetic (NOD) mouse used in this paper is a model of type 1 diabetes, but the authors mention other strains that would be very interesting in similar model systems, such as NONcNZO10/LtJ, a new mouse strain developed for studies of type 2 diabetes.[4] The Klueh implanted mouse model will permit new research that defines typical glucose excursions through the day in healthy animals and those

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with diabetes. This can help solve some questions about how to interpret continuous glucose monitoring and improve modeling of glucose regulation. These are critical questions to the further development and acceptance of continuous glucose monitors.[5] The model provides an efficient approach to testing new therapies, and the implanted system will itself be a test model for improvements in implantable systems, including studies of the interactions of implants and specific genetic traits and physiological conditions. This latter use could also be important in mitigating risk for the technology developer so that any differences between healthy individuals and patients with diabetes in host defense responses to an implant are identified and resolved early in the process. This model can also help to validate the applicability of specialized mouse strains to the development and progression of diabetes in humans, improving our confidence in biofidelic models of the human disease to further enable translational research.

Klueh and her colleagues are the poster children for effective leveraging. They have taken advantage of combined resources and talents in a collaboration between academia and industry, and availed themselves of some nontraditional government funding. This is a winning combination, according to the National Research Council's Committee on Accelerating Technology Transition.[1] From the perspective of the U.S. Army program in metabolic monitoring, methods development is highly relevant. Military research is oriented to pragmatic problem solving, and this changes the approach to extramural grants considerably from that of many other granting agencies. There is an impatience for solutions that will make a measurable difference, instead of a philosophy of supporting general discovery and the advancement of science. Thus, even when the Army funds basic research, it is focused on overcoming a technological barrier on the path to a military application. Development of an animal model is an example of this, where Klueh et al.[2] have provided a new tool to investigate the effects of militarily relevant stressors on metabolism. This can directly enhance prognostic and diagnostic capabilities in future physiological monitoring systems on the soldier. It can also help to understand points of intervention in performance-limiting metabolic pathways (e.g., critical times to increase carbohydrate consumption to sustain soldier mental or physical performance).

The model presented in this month's issue of *DT&T* must now be shown to be practical and reproducible in the hands of others. If the model of Klueh et al.[2] proves to be useful, we will hear more about it; significant methods papers tend to become highly cited references in the biomedical literature. The most important indicator of success will be that patients and soldiers realize the benefits of new discoveries in metabolic research sooner.

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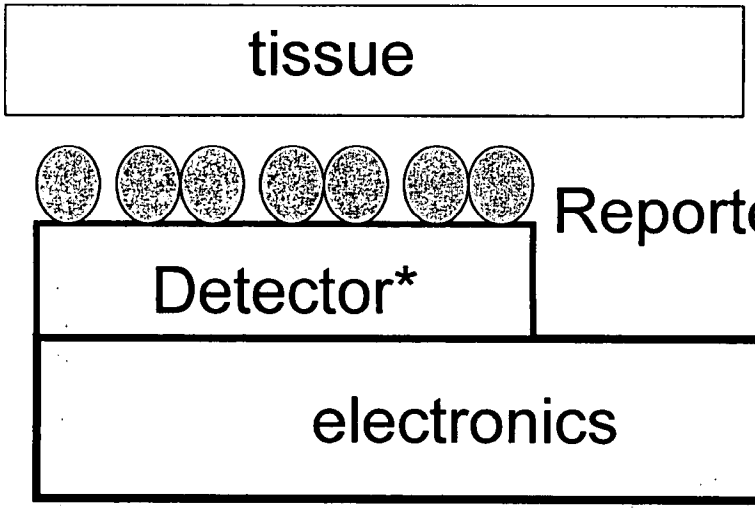
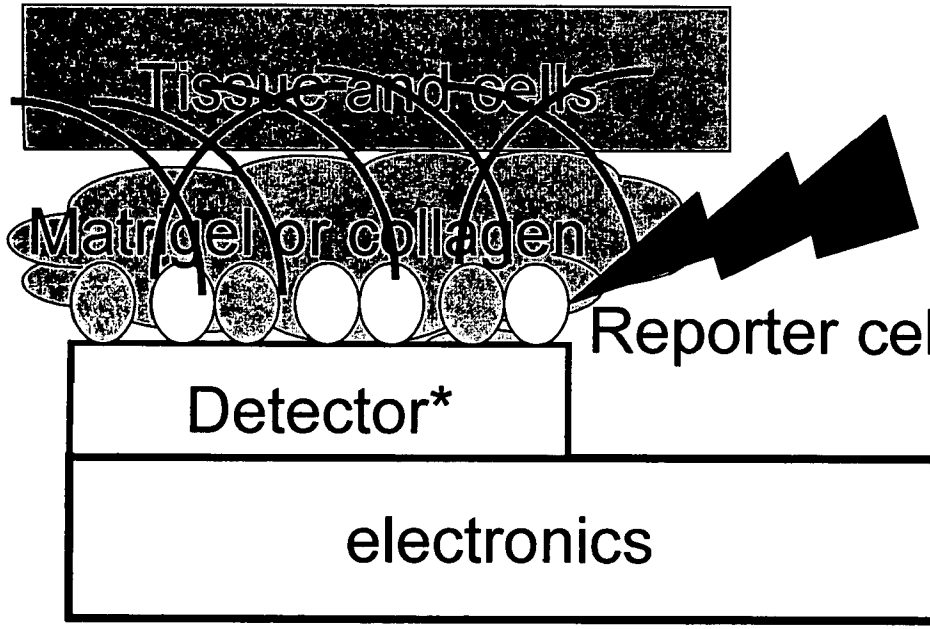
Exhibit 5



Device without protective membrane

Interference by autofluorescence, neovascularization and inflammation



Inflammation would destroy reporter cells & Dissolve matrix causing Reporter cells to be dislodged and the device would loss function

* Detector measures fluorescence (e.g. green colors)

Display Settings: Abstract

Kidney Int. 1993 Jan;43(1):147-50.

Basement membrane biosynthesis as a target for developing inhibitors of angiogenesis with anti-tumor properties.

Maragoudakis ME, Missirlis E, Karakiulakis GD, Sarmonica M, Bastakis M, Tsopanoglou N.

University of Patras Medical School, Department of Pharmacology, Greece.

Basement membrane (BM) exerts profound influence on endothelial cell (EC) behavior. In addition BM is a structural element of blood vessels; in fact at some point of their formation blood vessels are bare EC tubes lined with the BM produced by these EC. We thought, therefore, that a quantitative relationship must exist between the rate of BM synthesis and angiogenesis, and that interfering with BM synthesis must have an effect on angiogenesis. This was found experimentally in the chick chorioallantoic membrane (CAM) system. It was shown that the rate of BM collagen biosynthesis can serve as a biochemical index of angiogenesis and that inhibitors of BM synthesis prevent angiogenesis. GPA 1734 (8,9-dihydroxy-70-methyl-benzo (b)quinolizinium bromide), which inhibits proline and lysine hydroxylations in type IV collagen formation, suppresses angiogenesis in the CAM. Similarly, D609 (tricyclodecan-9-yl-xanthate), which inhibits BM synthesis by an as yet unknown mechanism, also prevents angiogenesis. Structurally related analogs of GPA 1734 and D609 that have no effect on BM biosynthesis are also without effect on angiogenesis. The aforementioned inhibitors of angiogenesis GPA 1734 and D609 have a dose-dependent inhibitory effect on tumor growth in rats bearing Walker 256 carcinosarcoma, without any obvious toxic effects. This effect is probably related to angiosuppression, since structurally related analogs that do not inhibit angiogenesis are without antitumor properties. Also GPA 1734 and D609 have no direct cytotoxic effects on Walker 256 cells in vitro. These results suggest that a search for agents that are specific inhibitors of BM synthesis may provide novel angiosuppressors with potential application in tumor chemotherapy.(ABSTRACT TRUNCATED AT 250 WORDS)

PMID: 7679456 [PubMed - indexed for MEDLINE]

Display Settings: Abstract

J Cell Physiol. 1992 Dec;153(3):614-25.

Interaction of endothelial cells with a laminin A chain peptide (SIKVAV) in vitro and induction of angiogenic behavior in vivo.

Grant DS, Kinsella JL, Fridman R, Auerbach R, Piasecki BA, Yamada Y, Zain M, Kleinman HK.

Laboratory of Developmental Biology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892.

Endothelial cells are known to bind to laminin, and two peptides derived from the laminin A (CTFALRGDNP) and B1 (CDPGYIGSR) chains block the capillary-like tube formation on a laminin-rich basement membrane matrix, Matrigel. In the present study, we have used various in vitro and in vivo assays to investigate the angiogenic-biologic effects of a third active site in the laminin A chain, CSRARKQAASIKVAVSADR (designated PA22-2) on endothelial cells. The SIKVAV-containing peptide was as active as the YIGSR-containing peptide for endothelial cell attachment but was less active than either the RGD-containing peptide or intact laminin. Endothelial cells seeded on this peptide appeared fibroblastic with many extended processes, unlike the normal cobblestone morphology observed on tissue culture plastic. In addition, in contrast to normal tube formation on Matrigel, short irregular structures formed, some of which penetrated the matrix and sprouting was more apparent. Analysis of endothelial cell conditioned media of cells cultured in the presence of this peptide indicated degradation of the Matrigel and zymograms demonstrated active collagenase IV (gelatinase) at 68 and 62 Kd. A murine in vivo angiogenesis assay and the chick yolk sac/chorioallantoic membrane assays with the peptide demonstrated increased endothelial cell mobilization, capillary branching, and vessel formation. These data suggest that the -SIKVAV-site may play an important role in initiating branching and formation of new capillaries from the parent vessels, a behavior that is observed in vivo in response to tumor growth or in the normal vascular response to injury.

PMID: 1280280 [PubMed - indexed for MEDLINE]

J Natl Cancer Inst. 1992 Nov 4;84(21):1633-8.

Role of the SIKVAV site of laminin in promotion of angiogenesis and tumor growth: an in vivo Matrigel model.

Kibbey MC, Grant DS, Kleinman HK.

Laboratory of Developmental Biology, National Institute of Dental Research, Bethesda, Md. 20892.

BACKGROUND: Angiogenesis (vascularization) has a critical role in tumor growth and metastasis, and peptides containing the SIKVAV amino acid sequence (Ser-Ile-Lys-Val-Ala-Val) have been shown to stimulate many angiogenic activities in vitro. The use of model systems to identify agents that stimulate or inhibit angiogenesis may lead to the development of new antitumor strategies. PURPOSE: Our purpose was to use an in vivo murine model system to study the angiogenic activity of a synthetic peptide derived from the laminin A protein chain and containing the SIKVAV amino acid sequence. We also examined the ability of the peptide to enhance tumor growth in vivo. METHODS: The SIKVAV-containing peptide was mixed with Matrigel, a reconstituted basement membrane extract used to assay stimulation of angiogenesis. The mixture was subcutaneously injected into C57BL/6 mice. At various times after injection, the Matrigel plug was excised, and angiogenic activity was assessed by histologic examination and immunohistochemical staining with an antibody to the von Willebrand factor (vWF), an endothelium-specific antigen. In other experiments, the mixture of peptide and Matrigel was co-injected with B16F10 murine melanoma cells into C57BL/6 mice, and the resultant tumors were assessed for size and vascularization. RESULTS: When co-injected with Matrigel at doses as low as 10 micrograms, the SIKVAV-containing peptide stimulated angiogenesis fourfold greater than that seen in controls, and maximum angiogenic activity was observed 2 weeks after injection. This peptide was angiogenic in a dose-dependent manner up to a 100-micrograms dose. When co-injected with Matrigel and B16F10 melanoma cells, the peptide enhanced tumor growth by approximately 2.5-fold, and tumor vascularization was significantly increased ($P = .027$) over that observed after injection with melanoma cells and Matrigel alone. CONCLUSIONS: These data demonstrate that the laminin-derived SIKVAV-containing peptide is angiogenic in a new in vivo model system and can enhance tumor vascularization and growth.

PMID: 1279183 [PubMed - indexed for MEDLINE]

Display Settings: Abstract

Lab Invest. 1992 Oct;67(4):519-28.

A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor.

Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, Pauly RR, Grant DS, Martin GR.

Laboratory of Biological Chemistry, National Institute on Aging, National Institutes of Health, Bethesda Maryland.

BACKGROUND: Blood vessel growth is necessary for normal tissue homeostatis and contributes to solid tumor growth. Methods to quantitate neovascularization should be useful in testing biological factors and drugs that regulate angiogenesis or to induce a vascular supply to promote wound healing. EXPERIMENTAL DESIGN: An extract of basement membrane proteins (Matrigel) was found to reconstitute into a gel when injected subcutaneously into C57/BL mice and to support an intense vascular response when supplemented with angiogenic factors. RESULTS: New vessels and von Willebrand factor antigen staining were apparent in the gel 2-3 days after injection, reaching a maximum after 3-5 days. Hemoglobin content of the gels was found to parallel the increase in vessels in the gel allowing ready quantitation. Angiogenesis was obtained with both acidic and basic fibroblast growth factors and was enhanced by heparin. Several substances were tested for angiostatic activity in this assay by coinjection in Matrigel with fibroblast growth factor and heparin. Platelet-derived growth factor BB, interleukin 1-beta, interleukin-6, and transforming growth factor-beta were potent inhibitors of neovascularization induced by fibroblast growth factor. Tumor necrosis factor-alpha did not alter the response but was alone a potent inducer of neovascularization when coinjected with Matrigel and heparin. Consistent with the previously demonstrated importance of collagenase in mediating endothelial cell invasion, a tissue inhibitor of metalloproteinases that also inhibits collagenases was found to be a potent inhibitor of fibroblast growth factor-induced angiogenesis. CONCLUSIONS: Our assay allows the ready quantitative assessment of angiogenic and anti-angiogenic factors and should be useful in the isolation of endothelial cells from the capillaries that penetrate into the gel.

PMID: 1279270 [PubMed - indexed for MEDLINE]

MeSH Terms, Substances

LinkOut - more resources

Nippon Geka Hokan. 1992 Mar 1;61(2):134-49.

[Relationship between endothelial cells and extracellular matrix: investigation using the model of angiogenesis in vitro]

[Article in Japanese]

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Department of Neurosurgery, Faculty of Medicine, Kyoto University, Japan.

Using model of angiogenesis in vitro, the relationship between endothelial cells and extracellular matrix was studied. Endothelial cells of bovine brain microvascular vessels (BBECs), carotid artery (BCECs) and aorta (BAECs) were cultured on type I collagen gel and Matrigel. BBECs make tubular structures and BCECs and BAECs grow and make confluent monolayer on type I collagen gel. BCECs and BAECs make tubular structures when second layer of collagen gel was overlaid. BBECs, BCECs and BAECs rapidly make tubular structures on Matrigel. These morphological changes were not affected by basic fibroblast growth factor. The effect of extracellular matrices on the cell kinetics and morphology of cultured bovine carotid artery endothelial cells was studied. Endothelial cells show a cobble stone appearance on plastic and type I collagen gel. They proliferate and make capillary-like structures on reconstituted gels composed of type I collagen and basement membrane substrata. And that endothelial cells forming capillary-like structures were increased with the increase in the concentration of basement membrane substrata. Transmission electromicroscopic examination study revealed endothelial cells forming capillary-like structures have junctional complexes on type I collagen and its mixture with basement membrane substrata. But endothelial cells on basement membrane substrata have no junctional complexes. These results suggest that BBECs have more potent angiogenic ability than BCECs and BAECs. And that proliferation and morphogenesis of endothelial cells are regulated by extracellular matrices.

PMID: 1382405 [PubMed - indexed for MEDLINE]

Microvasc Res. 1991 Nov;42(3):255-65.

Extracellular matrix of newly forming vessels--an immunohistochemical study.

Jerdan JA, Michels RG, Glaser BM.

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During wound healing, embryological development, and solid tumor growth, the established vasculature gives rise to large numbers of new blood vessels. This neovascular response occurs at the level of the capillary bed, where endothelial cells divide rapidly, locally remodel the surrounding stroma, and migrate away from existing vessels to form capillary sprouts. In order to examine the environment of these newly forming vessels, actively growing blood vessels in neovascularized rabbit and guinea pig corneas were examined immunohistochemically using antibodies against laminin, type IV collagen, heparan sulfate proteoglycans, entactin, and factor VIII-related antigen. Sequential serial 5-microns sections taken from the unfixed frozen corneas in a plane perpendicular to the direction of vessel growth were stained with these antibodies. It was possible to follow well-defined lumenized vessels out through sequential sections to the point where they became single factor VII-R positive cells in the region of the capillary sprout. Examination of these stained sections has shown the presence of four important basement membrane components--laminin, type IV collagen, heparan sulfate proteoglycan, and entactin--associated with actively migrating and invading capillary sprouts. These results suggest that the extracellular matrix of the actively invading capillary sprouts does not differ qualitatively from that of the established vasculature.

PMID: 1779882 [PubMed - indexed for MEDLINE]

Eye (Lond). 1991;5 (Pt 2):170-80.

Neovascular growth factors.

Schultz GS, Grant MB.

Department of Obstetrics, University of Florida, Gainesville 32610.

Neovascularisation is the biological process of forming new blood vessels. Many conditions can initiate neovascularisation including trauma or chronic ischaemia produced by diseases such as diabetes. Neovascularisation proceeds through a series of steps beginning with destruction of the basement membrane surrounding the microvascular endothelial cells, which allows endothelial cells to extend cytoplasmic buds in the direction of chemotactic factors. Migrating endothelial cells elongate, divide and eventually form tube structures which join to form mature new capillaries. Results of in vitro experiments, in vivo experiments, and clinical studies suggest that peptide growth factors can play key regulatory roles in each step of neovascularisation through both direct and indirect actions. At sites of vascular injuries, degranulating platelets release PDGF, IGF-I, EGF, and TGF-beta. Macrophages and neutrophiles drawn into the ischaemic or injured areas synthesise and release TGF-alpha, TGF-beta, and PDGF, and wounded endothelial cells secrete FGF. These peptide growth factors can stimulate migration, mitosis and differentiation of endothelial cells in culture and can induce neovascularisation in animal models. Clinical correlations suggest that peptide growth factors in the vitreous such as IGF-I and bFGF may promote diabetic retinopathy. As the biological mechanisms of neovascular growth factors become better understood, it may be possible to develop therapeutic approaches to selectively inhibit the peptide growth factors which regulate neovascular diseases.

PMID: 1712736 [PubMed - indexed for MEDLINE]

In Vitro Cell Dev Biol. 1990 Feb;26(2):119-28.

Modulation of microvascular growth and morphogenesis by reconstituted basement membrane gel in three-dimensional cultures of rat aorta: a comparative study of angiogenesis in matrigel, collagen, fibrin, and plasma clot.

Nicosia RF, Ottinetti A.

Department of Pathology, Medical College of Pennsylvania, Philadelphia 19129.

Rings of rat aorta cultured in Matrigel, a reconstituted gel composed of basement membrane molecules, gave rise to three-dimensional networks composed of solid cellular cords and occasional microvessels with slitlike lumina. Immunohistochemical and ultrastructural studies showed that the solid cords were composed of endothelial sprouts surrounded by nonendothelial mesenchymal cells. The angiogenic response of the aortic rings in Matrigel was compared to that obtained in interstitial collagen, fibrin, or plasma clot. Morphometric analysis demonstrated that the mean luminal area of the microvascular sprouts and channels was significantly smaller in Matrigel than in collagen, fibrin, or plasma clot. The percentage of patent microvessels in Matrigel was also markedly reduced. Autoradiographic studies of 3H-thymidine-labeled cultures showed reduced DNA synthesis by developing microvessels in Matrigel. The overall number of solid endothelial cords and microvessels was lower in Matrigel than in fibrin or plasma clot. A mixed cell population isolated from Matrigel cultures formed a monolayer in collagen or fibrin-coated dishes but rapidly reorganized into a polygonal network when plated on Matrigel. The observation that gels composed of basement membrane molecules modulate the canalization, proliferation, and organization into networks of vasoformative endothelial cells in three-dimensional cultures supports the hypothesis that the basement membrane is a potent regulator of microvascular growth and morphogenesis.

PMID: 1690206 [PubMed - indexed for MEDLINE]

Ann Chir Gynaecol. 1990;79(2):65-71.

Fibronectin, laminin, and collagen types I, III, IV and V in the healing rat colon anastomosis.

Brasken P, Lehto M, Renvall S.

Department of Surgery, University of Turku, Finland.

Comment in:

Ann Chir Gynaecol. 1990;79(2):63-4.

The purpose of this work was to study normal anastomotic healing in the rat colon. The unprepared sigmoid colon was divided and a colo-colostomy performed using a one-layer inverting technique. Frozen sections were taken and studied immunohistologically with specific antibodies to fibronectin, laminin and collagen types I, III, IV and V. From day one onwards a strong fibronectin reaction was observed in the anastomosis, reaching maximum staining intensity on postoperative day five. Type III collagen and pericellular type V collagen were at first detected in the anastomosis on day two. From day three onwards all collagens studied and laminin were present in the repair tissue (laminin and type IV and V collagen in the regenerating capillary walls). Maximum immunofluorescence was observed on day seven and it remained on a high level throughout the study, except for fibronectin, which weakened gradually after the fifth postoperative day. The results indicate that healing of the colon anastomosis occurs by rapid accumulation of connective tissue components between the inverted leaves of the colonic wall, as also new capillaries consisting of the basement membrane components, type IV and V collagen as well as laminin, are formed.

PMID: 1696798 [PubMed - indexed for MEDLINE]

Display Settings: Abstract

Acta Pathol Jpn. 1988 Dec;38(12):1503-12.

Capillary growth from reversed rat aortic segments cultured in collagen gel.

Mori M, Sadahira Y, Kawasaki S, Hayashi T, Notohara K, Awai M.

Department of Pathology, Okayama University Medical School, Japan.

The process of angiogenesis from aortic segments turned inside out and embedded in collagen gel was studied. Two to three days after inoculation, fibroblastic cells migrated from both ends of the segments. Later, capillary sprouts also appeared from both ends of the segments but not from the outer surface, even though there was a covering of endothelial cells. If the outer surface was injured, capillaries sometimes appeared at the damaged site. This may suggest that endothelial cells have more affinity for basement membrane than collagen gel and that they migrate only from an injured site. Immunohistochemical staining demonstrated factor VIII-related antigen in the capillary structures but not in the fibroblastic cells. Electron microscopically, capillary lumina were lined with several endothelial cells, and fibroblastic cells had the characteristics of smooth muscle cells. Since these fibroblastic cells have been known to appear under angiogenetic conditions in vivo, they may play an important role in angiogenesis, and the present culture technique may be a useful model for studying this process.

PMID: 2467510 [PubMed - indexed for MEDLINE]

Publication Types, MeSH Terms, Substances

J Pharmacol Exp Ther. 1988 Feb;244(2):729-33

Inhibition of basement membrane biosynthesis prevents angiogenesis.

Maragoudakis ME, Sarmonika M, Panoutsacopoulou M.

Department of Pharmacology, University of Patras Medical School, Patras, Greece.

GPA1734, an inhibitor of BM collagen biosynthesis, was investigated in the CAM model system for its effect on angiogenesis. Evaluation of angiogenesis was performed by placing a thin plastic coverslip inscribed with concentric circles on the CAM and counting the number of vessels intercepting the circles. The rate of BM collagen biosynthesis was monitored using [U-14C] proline incorporation into CAM proteins and determining the collagenase-digestible protein fraction. A marked depression in the vascular density was observed in the CAM area under a plastic disc containing GPA1734 as compared to control discs placed on the CAM about 1 cm apart from days 9 to 12 of incubation. A concomitant decrease in collagenous protein biosynthesis was observed in the area under the discs containing GPA1734 and [U-14C]proline as compared to control discs containing only the radiolabeled proline. The forementioned effects of GPA1734 on CAM were specific because no similar effects were observed with a closely related compound, 9,10-dihydroxy-7-methyl-benzo[b]quinolizinium bromide or with GPA1734 plus Fe++, which did not affect the rate of BM collagen biosynthesis. These results suggest that inhibitors of BM collagen biosynthesis prevent angiogenesis by interfering with the formation of an essential component of the vessel wall. The search for such inhibitors may be a new approach in the development of antiangiogenic agents.

PMID: 2450202 [PubMed - indexed for MEDLINE]

Am J Pathol. 1988 Feb;130(2):393-400.

A heparin-binding angiogenic protein--basic fibroblast growth factor--is stored within basement membrane.

Folkman J, Klagsbrun M, Sasse J, Wadzinski M, Ingber D, Vlodavsky I.

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The basement membranes of bovine cornea are found to contain an angiogenic endothelial cell mitogen, basic fibroblast growth factor (FGF), as determined by heparin-affinity chromatography, immunoblotting, immunofluorescence, and stimulation of capillary endothelial cell proliferation. The growth factor appears to be bound to heparan sulfate and is released from the cornea by treatment with heparin, a hexasaccharide heparin fragment, heparan sulfate, or heparanase, but not by chondroitin sulfate or chondroitinase. These findings indicate that basement membranes of the cornea may serve as physiologic storage depots for an angiogenic molecule. Abnormal release of this growth factor could be responsible for corneal neovascularization in a variety of ocular diseases. Physiologic and pathologic neovascularization in other tissues may also be initiated by release of stored angiogenic factors from the basement membrane. The sequestration of angiogenic endothelial mitogens in the basement membrane may be a general mechanism for regulating their accessibility to vascular endothelium.

PMCID: PMC1880518

PMID: 3277442 [PubMed - indexed for MEDLINE]

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Tissue Cell. 1988;20(4):531-9.

Rate of basement membrane biosynthesis as an index to angiogenesis.

Maragoudakis ME, Panoutsacopoulou M, Sarmonika M.

Department of Pharmacology, University of Patras Medical School, Greece.

A method was developed for assessing collagenous protein biosynthesis from [U-14C]proline in relation to angiogenesis in the chick chorioallantoic membrane (CAM). The rate of collagenous protein biosynthesis both in vitro and in vivo was maximum between days 8 and 11 of chick embryo development. This was the stage of maximum angiogenesis as shown by morphological evaluation of the vascular density. At day 10 the rate of collagenous protein biosynthesis was 11-fold higher than that of day 15, when angiogenesis had reached a plateau. The collagenous protein formed by CAM co-elutes on SDS-agarose chromatography with the collagenous component of [3H]-acetylated-basement membrane (BM) from bovine lens capsule. 8,9-dihydroxy-7-methyl-benzo[b]quinolizinium bromide (GPA1734), which was shown previously to be a specific inhibitor of BM collagen biosynthesis, caused about 80% reduction in collagenous protein synthesis by CAM. These results indicate that most of the collagenous protein synthesized by CAM was BM collagen and this can be used as a biochemical index of angiogenesis.

PMID: 2467405 [PubMed - indexed for MEDLINE]

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Lab Invest. 1986 Nov;55(5):521-30.

Endothelial cell proliferation during angiogenesis. In vitro modulation by basement membrane components.

Form DM, Pratt BM, Madri JA.

Modulation of the behavior of microvascular endothelial cells during angiogenesis has been observed to correlate with changes in the extracellular matrix. These reports prompted a comparison of the growth of microvascular endothelial cells on monolayers of various matrix components in vitro. Over a 5 day period, the proliferation of these cells was significantly greater on laminin than on either plasma fibronectin, the interstitial collagen types I and III, or on the basement membrane collagen type IV. Proliferation of the microvascular endothelial cells was compared with that of bovine aortic endothelial cells and bovine aortic smooth muscle cells on the same matrices. All three cell types grew significantly more rapidly on laminin than on fibronectin. The aortic endothelial cells differed from their microvascular counterparts in that the growth of these large vessel endothelial cells on the collagenous matrices (types I and III, or type IV) was not significantly different from that observed for laminin, but was greater than the relatively slow growth seen on plasma fibronectin. Further comparison of the growth of the microvascular endothelial cells on the two basement membrane components, laminin and type IV collagen, demonstrated that the growth of these cells on laminin can be modulated by the presence of type IV collagen. This was true either if the two matrices were combined as a mixed layer, or if the laminin was specifically bound to a layer of type IV collagen, more closely simulating the distribution of these molecules in a basement membrane. Examination by immunoperoxidase of in vivo model of neovascularization in the murine cornea revealed a temporally staggered appearance of basement membrane components. The appearance of laminin was found to occur throughout the newly formed vessels, as well as in individual cells at the migrating, proliferating tips. In contrast, the appearance of type IV collagen correlated with lumen formation and was not detected at the vessel tips. The results of this study suggest that the temporally ordered synthesis of specific matrix components plays a significant role in orchestrating the growth and differentiation of endothelial cells during the highly integrated set of responses known as angiogenesis.

PMID: 2430138 [PubMed - indexed for MEDLINE]

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Br J Haematol. 1986 Jul;63(3):571-80.

Immunohistochemical study of basement membrane proteins and type III procollagen in myelofibrosis.

Apaja-Sarkkinen M, Autio-Harmainen H, Alavaikko M, Risteli J, Risteli L.

In this study the distribution of type IV collagen in the marrow is compared with that of laminin, another basement membrane protein. In addition, incompletely processed type III procollagen is identified with specific antibodies. In normal bone marrow the distribution of the type III procollagen antigen closely resembles that of reticulin staining. In all the myelofibrotic samples, representing both early and advanced disease, the fibrous tissue stains heavily for this antigen. Thus type III procollagen which has not completely lost its aminoterminal propeptide is a genuine component of the extracellular matrix fibres in human bone marrow. Laminin is found with type IV collagen in continuous basement membranes in arterial walls, whereas only discontinuous strips of staining are seen along the sinusoids in normal marrow. In myelofibrosis the dilated or obliterated sinusoids have thickened or continuous basement membranes, visible with both stainings. Neovascularization also increases the extent of basement membrane staining in fibrotic marrow. With respect of these antigens, there is no difference between primary and secondary myelofibrosis. These changes warrant the use of serum antigens related to type IV collagen and to type III procollagen as markers for developing myelofibrosis.

PMID: 3524658 [PubMed - indexed for MEDLINE]

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Science. 1983 Jul 15;221(4607):281-3.

Basement membrane collagen: degradation by migrating endothelial cells.

Kalebic T, Garbisa S, Glaser B, Liotta LA.

One of the first steps in neovascularization is dissolution of the basement membrane at the point of endothelial outgrowth. An assay was developed to determine whether basement membrane collagens (types IV and V) are degraded by endothelial cells migrating toward a chemotactic stimulus. Fetal bovine endothelial cells were placed on one side of a filter containing the collagen substrate, and a chemoattractant derived from retinal extracts was placed on the opposite side. Degradation of both type IV and type V collagens was observed when the retinal factor was placed on the side of the filter opposite the endothelial cells. Metalloproteinases that cleaved type IV and type V collagens could be extracted from the endothelial cells with detergents. Such endothelial cell-associated (possibly membrane-bound) proteinases may locally disrupt the basement membrane and facilitate the outgrowth of capillary sprouts toward the angiogenic stimulus.

PMID: 6190230 [PubMed - indexed for MEDLINE]

MeSH Terms, Substances

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Ciba Found Symp. 1983;100:150-62.

Degradation of basement membrane components by vascular endothelial cells: role in neovascularization.

Glaser BM, Kalebic T, Garbisa S, Connor TB Jr, Liotta LA.

A retina-derived substance (or substances) has been identified that stimulates vascular endothelial cells to degrade three structural components of basement membrane: type IV collagen, fibronectin and laminin. In basement membrane surrounding existing blood vessels, endothelial cells stimulated in this way can presumably migrate through the resulting gaps and form new vascular sprouts. These events may therefore represent the earliest steps in the formation of new blood vessels.

PMID: 6197258 [PubMed - indexed for MEDLINE]

MeSH Terms, Substances